Aspects of the physiological ecology of the Western-Australian ruderal orchid, Microtis media R.Br, with special reference to the functions of its mycorrhizal fungi

Wei-Han Lim
BSc (Hons), MPhil

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Abstract

This study focuses on the common mignonette orchid, *Microtis media* (R.br), which in recent decades, has successfully expanded beyond its natural habitats, often into novel and disturbed settings such as garden and horticultural beds, and will explore aspects of the physiology of *M. media* that may have contributed to the success of the orchid. Particular emphasis was placed on elucidating the functionality of mycorrhizal fungi associating with *M. media*.

Investigations into the basic eco-physiology of *M. media* have highlighted the lack of reproductive constraint within the species, with seed production occurring through autogamy and allogamy, and vegetative reproduction occurring through the formation of daughter tubers. It was also determined that there were no barriers to *M. media* seed germination, with the lack of photo-inhibition on germination, and the occurrence of asymbiotic seed germination on water agar. However, the lack of normal development in these asymbiotic seedlings also indicated that associations with mycorrhizal fungi were essential in order to allow for the normal development of *M. media* protocorms.

Characterizing the symbiotic germination process of *M. media* highlighted the extremely rapid rate of *M. media* protocorm development, after being colonized by an appropriate mycorrhizal symbiont. Within four weeks of sowing, *M. media* seeds developed into photosynthetic stage five protocorms, and the *in-vitro* flowering of multiple symbiotic seedlings was observed by 16 weeks of age, often followed with the formation of seeds by autogamy. This accelerated development also shows that *M. media* is able to mature rapidly in the presence of a compatible fungal partner under optimal conditions, which may explain the rapid proliferation observed in novel settings.

Molecular identification of mycorrhizal fungi associating with *M. media* revealed that the orchid predominantly associated with species of the fungal genus *Tulasnella*, which are cosmopolitan soil saprophytes and orchid symbionts. Despite being from the same genus, the *Tulasnella* isolates of this study varied in their abilities to promote *M. media* germination and growth, potentially reflecting differences in ecological functionality.
The ability to associate with multiple Tulasnella isolates has the potential to allow for the ruderal expansion of *M. media* if these are widespread mycorrhiza. The proliferation of *M. media* in the modified mulched environments of garden beds would suggest that not only is Tulasnella a genus of disturbance tolerant fungi, but that the formation of a symbiosis with *M. media* is also a robust process in disturbed areas.

Investigations of the phosphorus (P) physiology of *M. media* revealed evidence of preferential accumulation of the element in its seeds, which can be inferred to be an adaptation to maximise seedling survivability. Demonstration of the ability of the mycorrhizal symbiont of *M. media* to utilise both organic and inorganic forms of the element, and to facilitate the transport of P to the developing protocorms and to adult plants establishes the likely functional importance of the symbiosis in the essentially P-starved natural environments colonized by the orchid.

Analysis of carbon flows in *M. media* demonstrated the bidirectional flow of nutrients in the orchid-mycorrhizal symbiosis. ‘Up-flow’ of carbon (assimilated from cellulose) from mycorrhizal fungi to developing protocorms of *M. media* was established. In addition, the ‘down-flow’ of carbon (fixed by photosynthesis) from orchid to mycorrhizal fungi establishes the bi-directional movement of nutrients between *M. media* and its mycorrhizal symbiont, and is indicative of the mutualistic nature of this symbiosis. This study also demonstrated the inter-generational sharing of carbon resources in *M. media*, where carbon assimilated by photosynthesizing adults is transferred or shared with non-photosynthetic protocorms by way of the mycorrhizal symbiont.

It is concluded that the fungal pathways identified in this study should contribute significantly to the ability of the orchid to exploit the minimal phosphorus reserves in bushland soil. It is proposed that the mycorrhizal association may thus provide a fundamental contribution to the success of *M. media* as a colonist of both natural and disturbed soil systems throughout Western-Australia. Further studies will be required to determine the relative importance of other attributes of its biology, in particular the shortening of the period between germination to seed-set, and aspects of its pollination biology.
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This thesis is dedicated to my late grandparents, who greatly encouraged me onto this path of scientific research. Finally, I would like to thank my parents for their continual love and support, which has made the past few years of living away from home bearable.
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Declaration of candidate contribution

DECLARATION FOR THESES CONTAINING PUBLISHED WORK AND/OR WORK PREPARED FOR PUBLICATION

The examination of the thesis is an examination of the work of the student. The work must have been substantially conducted by the student during enrolment in the degree.

Where the thesis includes work to which others have contributed, the thesis must include a statement that makes the student’s contribution clear to the examiners. This may be in the form of a description of the precise contribution of the student to the work presented for examination and/or a statement of the percentage of the work that was done by the student.

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Signature........................................................................................................................................

Coordinating Supervisor
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Chapter 1: General Introduction
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1.1 Introduction

The orchid *Microtis media* (R.Br) occurs as a natural component of the highly diverse plant communities of the South West Australia Floristic Region (SWAFR – see Chapter 5), and as an invasive ruderal in disturbed habitats of the region. The work described in this thesis was designed to investigate the biological basis of the success of this orchid species, which grows in some of the world’s most nutritionally impoverished soils (McArthur, 1991; Lambers, 2014).

Amongst the diverse range of plant species that have successfully colonised these soils, a number of specialized adaptations to the prevailing nutritional limitations can be seen. Each of these adaptations is primarily concerned with greater nutrient acquisition in these nutrient depauperate soils. The most frequently observed adaptation of plant structure, seen throughout the Proteaceae, Restionaceae and Cyperaceae, involves the production of repeatedly branched ‘cluster roots’, which are involved in the production and exudation of carboxylates, which facilitates the solubilisation of the major growth limiting element phosphorus (P) (Shane *et al.*, 2004; Lambers *et al.*, 2006). In addition to specializations involving changes of root anatomy, a number of the characteristic species of the SWAFR, including the orchids, form associations with symbiotic fungi to form mycorrhizas.

At the global level, seven major mycorrhizal types are recognized (Table 1.1). Amongst these, while significant nutritional benefits to the host plant have been extensively shown to be based upon fungal colonization in the arbuscular, ectomycorrhizal and ericoid mycorrhizal types (Finlay & Read, 1986; Jakobsen *et al.*, 2001; Van Leerdam *et al.*, 2001; van Schöll *et al.*, 2008; Martino & Perotto, 2010), relatively little information concerning the possible role of mycorrhizas in the mineral nutrition of orchids have emerged, and only a few investigations have been carried out on the nutrition of Australian orchids (Bougoure *et al.*, 2010; Dearnaley & Bougoure, 2010; Sommer *et al.*, 2012; Bougoure *et al.*, 2014).
Table 1.1 Characteristics of the seven major mycorrhizal types. The structural characters given relate to the mature state, not the developing or senescent states. Entries in brackets indicate rare conditions. Adapted from Smith and Read (2008).

<table>
<thead>
<tr>
<th>Mycorrhizal types</th>
<th>Arbuscular mycorrhiza</th>
<th>Ectomycorrhiza</th>
<th>Ectendomycorrhiza</th>
<th>Arbutoid mycorrhiza</th>
<th>Monotropoid mycorrhiza</th>
<th>Ericoid mycorrhiza</th>
<th>Orchid mycorrhiza</th>
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<tr>
<td>Fungi: Septate</td>
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<td>+</td>
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<td>Fungal mantle</td>
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<td>+ or -</td>
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<td>Hartig net</td>
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<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Fungal taxa</td>
<td>Glomero Basidio/Asco</td>
<td>Basidio/Asco</td>
<td>Basidio Asco</td>
<td>Basidio</td>
<td>Basidio</td>
<td>Asco</td>
<td>Basidio</td>
</tr>
<tr>
<td>Plant taxa</td>
<td>Bryo Gymno Pterido</td>
<td>Angio Gymno</td>
<td>Angio Ericales</td>
<td>Monotropoideae</td>
<td>Ericales</td>
<td>Orchidales</td>
<td>Bryo</td>
</tr>
<tr>
<td></td>
<td>Gymno Angio</td>
<td>Angio</td>
<td>Angio Ericales</td>
<td>Monotropoideae</td>
<td>Ericales</td>
<td>Orchidales</td>
<td>Angio</td>
</tr>
</tbody>
</table>

All orchids are achlorophyllous in the early seedling stages. Most orchids are green as adults. The fungal taxa are abbreviated from Glomeromycota, Ascomycota and Basidiomycota; the plant taxa from Bryophyta, Pteridophyta, Gymnospermae and Angiospermae.
Mycorrhizal associations or symbioses are defined as intimate relationships between fungi and the nutrient absorbing organs of plants, and range from being mutualistic; where both parties benefit from being in the association, to parasitic; where only one party benefits from the association, often with detrimental effects on the other (Harley & Smith, 1983). The forming of mycorrhizal associations between early land-based fungi and early plants, which were lacking in ‘true roots’, has been suggested as one of the main events leading to the successful colonization of land by plants (Pirozynski & Malloch, 1975; Simon et al., 1993; Kenrick & Crane, 1997; Brundrett, 2002). The discovery of fungal ‘arbuscule’- like structures within the below ground structures of *Aglaoophyton major*, an early Devonian fern-like plant, has led to two main hypotheses; that plant/mycorrhizal fungi associations have existed for a minimum of 400 million years (Pirozynski & Malloch, 1975; Remy et al., 1994; Kenrick & Crane, 1997; Brundrett, 2002; Taylor et al., 2014), and that the mutualistic transfer of nutrients between plants and fungi could have been in existence at the very early stages of land colonization (Remy et al., 1994; Taylor et al., 2014).

Although mycorrhizal structures within orchid roots were reported as early as 1847 (Reissek, 1847), it was not until between 1899 (Bernard, 1899) and 1909 (Burgeff, 1909) that they were formally described as pelotons; intracellular hyphal coils, which is now recognised as one of the definitive features of orchid mycorrhizas (Smith & Read, 2008). Studies have established that the majority of orchid mycorrhizas belong to the polyphyletic form genus Rhizoctonia, within the Basidiomycota, and is comprised of a disparate group of distantly related fungal clades belonging to three different fungal families: Ceratobasidiaceae, Tulasnellaceae and Sebacinaeaceae (Rasmussen, 2002; Taylor et al., 2002; Suárez et al., 2006; Bonnardeaux et al., 2007; Brown et al., 2008; Smith & Read, 2008; Dearnaley et al., 2012; Pereira et al., 2014). While most of these fungi are able to form symbiotic associations with orchids, they are also able to persist independently in nature as saprotrophs (Clements, 1988; Bonnardeaux et al., 2007; Nurfadilah et al., 2013). In addition, some ascomycete fungi have been reported to be involved in symbiotic associations with orchids (Bidartondo et al., 2004; Selosse et al., 2004; Ogura-Tsujita & Yukawa, 2008; Ogura-Tsujita et al., 2009; Roy et al., 2009; Dearnaley et al., 2012).
While the mutualistic nature of orchid mycorrhizal symbioses has been demonstrated in a few Northern-hemisphere orchids (Cameron et al., 2006; Cameron et al., 2007; Cameron et al., 2008; Liebel et al., 2010; Kuga et al., 2014), relatively little is known about the nature and functionality of mycorrhizal fungi in green Australian orchids. Mycorrhizal functionality, in terms of the provision of nutrients such as carbon and nitrogen to the orchid, has been confirmed in two Australian achlorophyllous species, *Gastrodia sesamoides* R.Br and *Rhizanthella gardneri* Rogers (Bougoure et al., 2010; Dearnaley & Bougoure, 2010; Bougoure et al., 2014). In addition, although six out of the 27 Australian orchid species investigated by Sommer et al. (2012) were shown to be partially or fully myco-heterotrophic, the nature and functionality of mycorrhizal fungi associating with the remaining 21 orchid species remains undetermined, primarily due to the larger than expected variation in the natural isotopic abundance of co-occurring non-orchid reference plants.

Within Australia, an estimated 192 genera and approximately 1700 orchid species have been reported, with up to 95% of species being endemic (Jones, 1988). Specifically, an estimated 28 genera comprising approximately 394 orchid species, all of which are terrestrials with the exception of one true epiphyte; *Dendrobium dicuphum* (F.Muell), have been reported within Western-Australia, with most of these orchids occurring within the SWAFR (Brown et al., 2008; Hoffman & Brown, 2011; Brown et al., 2014). Being deciduous, many of the species survive the dry season (Kimberley region – winter, SWAFR – summer) by senescing back to underground tubers or rhizomes, and are re-colonized by mycorrhizal fungi annually following regrowth (Brown et al., 2008; Hoffman & Brown, 2011; Brown et al., 2014).

Five major patterns of fungal colonization (Ramsay et al., 1986) are recognized in West-Australian terrestrial orchids (see Chapter 3); stem tuber – where mycorrhizal colonization is confined to the swollen underground stems; underground stem – where mycorrhizal colonization occurs in the vertical underground stem (between the tuber and stem collar); stem collar – where heavy mycorrhizal colonization occurs in a swollen region 3 to 10 mm long, just at or below soil surface; root – where mycorrhizal colonization is confined to the roots; and root/stem – where mycorrhizal colonization occurs concurrently in both the stem collar and within roots. Mycorrhizal colonization of the study species, *M. media*, occurs within the roots (Ramsay et al., 1986).
As in all orchids, the reproductive process in *M. media* involves the production of vast numbers of minute seeds (Arditti & Ghani, 2000; Brown et al., 2008). Members of the Orchidaceae (as well as a small number of other mostly myco-heterotrophic or parasitic plant families which also show the extreme reduction of seed size) have been able to achieve this diminution by adopting a life-style in which the essentially reserve-free ‘seedling’ is assured of an external source of support from the very earliest stages of germination, by selectively forming associations with specific fungal symbionts (Arditti, 1967; Rasmussen, 1995). This removes the need for seed nutrient reserves. Further, the constraint on seed numbers that is imposed upon most plants by the need for the parent plant to divert resources for the provisioning of seed is also removed. Under this distinctive set of biological circumstances, the overall reproductive ‘fitness’ of a plant species can be improved by increasing the number of progeny. Clearly, the success of such a strategy is entirely dependent upon the availability of this external source of support.

By selectively forming partnerships with a group of basidiomycetous fungi in the form genus *Rhizoctonia* (see Table 1.1), which occurs perhaps universally as an occupant of natural and cultivated soils, terrestrial orchids have optimised the chances that their seeds will gain access to a compatible fungal symbiont (Smith & Read, 2008). It may at least be in part, a testament to the success of this strategy that the Orchidaceae has become one of the largest family of flowering plants on earth, with an estimated 25000 species (Chase, 2001).

While the studies of pioneers such as Bernard (1899) and Burgeff (1909) did much to elucidate the structural and broad taxonomic relationships within orchid mycorrhizal systems (Burgeff, 1959), and the structural features of orchid seeds have been described (Arditti & Ghani, 2000), relatively little is known about the physiological ecology of these plants. The early provisioning of nutrients (by their mycorrhizal symbionts) in germinating orchid seeds remain largely undescribed, and studies of the mechanisms whereby orchid-fungal systems explore the soil for mineral nutrients remain in their infancy.

Specifically, in the case of *M. media*, it is of interest to learn whether there are any unique biological features of the reproductive cycle that might contribute to the
success of the orchid as an invasive ruderal species. In addition to prolific seed production, a further possible adaptation to a ruderal life-style would be the shortening of the period between germination and seed production, relative to that seen in non-ruderal orchids. Likewise, any mechanisms that enhance the provisioning of nutrients to the seed, in particular carbon (C), which is in quantitative terms, the major nutrient required by the germinating seed, could be of considerable biological significance. These aspects of the biology of *M. media* are considered in Chapters 2, 3 and 6 of this thesis.

In addition to the provision of C through the early, non-photosynthetic stage of development of the orchid, the plant has a life-long requirement for the supply of mineral nutrients. Orchids, in general, are distinguishable from almost all other plants by the paucity of their root development (Leake, 1994; Brundrett, 2002). Based upon the observation that orchid protocorms generally lack roots, and that fibrous root systems are never developed even in adult orchids, some authors (Rasmussen, 1995; Brundrett, 2002) have chosen to make the assumption that the fungal symbionts must be involved in nutrient acquisition. However, experimental confirmation of such functional roles has mostly been lacking, with the exception of a few studies (see above).

In the context of mineral nutrition, the contribution of mycorrhiza to the success of *M. media*, both as an inhabitant of natural bushland soils, and as an invasive colonizer of disturbed sites, is of particular interest. Amongst the mineral elements that can be predicted to be the most critical for any plant will be those that: a) fundamentally underpin the physiology of the plant, and b) are of lowest availability in the natural habitats of the plant in question. In the case of *M. media*, this element is phosphorus (P). For this reason, particular emphasis in this thesis has been placed upon the role of mycorrhizal fungi associating with *M. media*, in the capture and transfer of P to the orchid (Chapter 5).

Phosphorus, an essential plant macronutrient of soil origin, is estimated to make up between 0.15 to 0.25 % of a plant’s dry weight (Marschner, 1995; Schachtman *et al.*, 1998; Taiz & Zeiger, 2010). It is a constituent of essential molecules such as nucleic acids (DNA and RNA) and phospholipids (Schachtman *et al.*, 1998; Marschner &
Within plant cells, phosphate esters (mostly functioning as intermediates within metabolic pathways) and phosphatases (mostly present as adenosine tri-phosphate (ATP), which ‘fuels’ most metabolic pathways such as carbohydrate syntheses) are intrinsically involved in the regulation and fuelling of metabolic pathways (Schachtman et al., 1998; Taiz & Zeiger, 2010; Marschner & Marschner, 2012).

The availability of P to plants is influenced by several factors (Figure 1.1, phosphorus cycle within soils), and although P is present within soil in both organic and inorganic forms, it is the inorganic orthophosphate form which is directly taken up by plant roots (McArthur, 1991; Schachtman et al., 1998). Animal, plant and microbial remains constitute significant sources of organic P (such as inositol phosphates, phospholipids and nucleic acids) within soil (van Diest, 1968; Bieleski, 1973), but these are thought to remain largely unavailable to plants unless mineralized by extracellular exudates such as phosphatases into inorganic forms (Schachtman et al., 1998; Peverill et al., 1999).

Thus, although P present within the soil solution constitutes less than 1 % of total P, most plants derive their P requirements from the soil solution (Barber, 1984; Bolan, 1991; Marschner, 1995). As highlighted by Barber (1984), given that P is readily adsorbed by soil particles, its movement, predominantly by diffusion, through soils is very slow when compared to other nutrients. This often results in the formation of zones of P depletion around plant roots, as the rates of plant P uptake are much quicker than the rates of replenishment (Bieleski, 1973; Barber, 1984; Marschner, 1995).
While the concentrations of P between the soil solution and adsorbed P on soil aggregates are generally thought to be in equilibrium (Bolan, 1991), studies investigating P concentration differences between these two pools have shown that it is the labile portion of adsorbed P which is drawn upon to equilibrate the differences within the soil solution, indirectly demonstrating that the labile portion of adsorbed P is available to plants (Amer et al., 1955; Vaidyanathan & Talibudeen, 1970). In contrast, the fixed or non-labile P, formed where P ions, over an extended period of time, have migrated deep into the micro-pores of soil aggregates, or have precipitated into discreet minerals, remain largely inaccessible to plants, unless solubilised by extracellular exudates such as organic acids (carboxylates) and phosphatases (Bieleski, 1973; Bolan, 1991; Raghothama, 1999).

The inherent inaccessibility of the phosphate ion in the terrestrial environment must have presented strong selective forces favouring the formation of mycorrhizal associations between plant roots and fungi. Theoretical considerations suggest that fungal hyphae, with their potentially rapid growth rates and inherently narrow diameters, have the potential to forage beyond the depletion zones around roots, and
thus explore for P in microhabitats at considerable distances from the root surface (Harley, 1989; Marschner & Dell, 1994; Peverill et al., 1999). Further, as heterotrophs which have evolved under soil conditions, many fungi can be expected to have the capability to mobilise nutrient elements such as P from organic sources in soil.

When analysing the functional basis of the most ancient form of arbuscular-type mycorrhiza, Baylis (1975) hypothesized that plants most likely to benefit from such symbiotic associations with fungal heterotrophs would be those that possessed coarse roots, as opposed to plants with fibrous and fine root systems. Coarse and tuberous root systems develop the largest nutrient depletion zones and have the smallest absorptive surface to volume ratio (Baylis, 1975). While the hypothesis put forward by Baylis (1975) has been largely confirmed with plants associating with arbuscular mycorrhizal fungi, it has scarcely been tested in the field of orchid mycorrhiza. This is particularly surprising in view of the fact that the Orchidaceae, while being arguably one of the most successful plant families on Earth, has achieved this success despite having the least fibrous of root systems. In this context, *Microtis media*, the orchid selected for study in this thesis may provide a particularly useful model to test the applicability of some aspects of the Baylis (1975) hypothesis on orchids. This thesis will explore the aspects of the biology of *M. media* that may contribute to the success of the orchid, paying particular attention to the role played by its mycorrhizal symbionts in enabling it to complete its life cycle.
1.2 Aims

The overarching aim of this thesis is to conduct in-depth studies into the eco-physiological attributes of a common and ruderal terrestrial orchid found within Western-Australia, *Microtis media*. Specifically, experiments were designed to test the following hypotheses:

- **Hypothesis 1**: That multiple aspects of the broader asymbiotic eco-physiology of *M. media* contribute to its success in its natural environment (Chapter 2).
- **Hypothesis 2**: That aspects of the symbiotic germination process will play a key role in determining the ability of *M. media* to establish in its natural environments (Chapter 3).
- **Hypothesis 3**: That particular taxonomic and eco-physiological attributes of the mycorrhizal fungal associates of *M. media* will promote seed germination and successful establishment under *in-vitro* and *in-situ* conditions (Chapter 4).
- **Hypothesis 4**: That the mycorrhizal fungus/fungi play a central role in the provision of the growth-limiting element, phosphorus, to the developing orchid (Chapter 5).
- **Hypothesis 5**: That the mycorrhizal fungi of *M. media* are involved -
  
  a) As donors of carbon in the below-ground heterotrophic (protocorm) phases of orchid development (Chapter 6).
  
  b) As recipients and translocators of C from the adults in their autotrophic phases of growth, to the soil, and potentially to developing heterotrophic protocorms (Chapter 6).
1.3 References


Chapter 2: Some non-symbiotic eco-physiological attributes of the ruderal orchid, *Microtis media*
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2. Abstract

*Microtis media*, a native West-Australian terrestrial orchid, has been actively expanding beyond its natural distributions in the recent decades. Investigations into the eco-physiological (non-symbiotic) characteristics of *M. media* have established attributes which have contributed to the ruderal habitat of the species. The apparent lack of reproductive constraint was established for *M. media*; with seed production occurring by both autogamy and allogamy, and vegetative reproduction occurring by the production of daughter tubers. The presence of circular openings along the seed coats of *M. media* seeds, established by ultra-structure investigations, can be inferred as adaptations facilitating seed germination by increasing seed coat permeability. In addition, investigations into the germination of *M. media* seeds have established the non-fastidious requirements of the species, with *M. media* seeds germinating and developing asymbiotically to a green stage (somewhat atypically) on water agar. The lack of photo-inhibition suggests the inherent ability of *M. media* seeds to germinate both at the soil surface, and at depth. However, the atypical development of these asymbiotic seedlings also suggests that mycorrhizal associations are essential to allow for normal *M. media* development. All of the abovementioned adaptations are fitting of a ruderal species, and would have contributed to the successful expansion of *M. media*.

2.1 Introduction

*Microtis* R.Br (Greek *mikros* - small, and *ous, otos* - ear), a genus of slender herbaceous orchids, was first described by Robert Brown in 1810 (Brown, 1810; Jones, 1988; Brown et al., 2008). The genus consists of 21 terrestrial species spread across a wide longitudinal range (Figure 2.1a, from 36° north in Japan to 47° south on Stewart Island in New Zealand), and have been reported to occur from coastal lowlands to montane highlands (encompassing temperate, subtropical and tropical-highland regions) (Jones, 1988; Pridgeon et al., 2001; Brown et al., 2008). The greatest diversity of the genus occurs within Australia, with 18 species occurring between 15° 30’ south near Cooktown (Queensland) and approximately 43° south in Tasmania (Pridgeon et al.,
Within Western Australia, *Microtis* is well represented, with ten formally named species (Pridgeon et al., 2001; Brown et al., 2008).

All *Microtis* species share two common characteristics: the occurrence of small columnar wings (resembling ears) flanking the anthers of the flowers, and shoots comprising of a single tapering, terete and hollow leaf (Brown, 1810; Jones, 1988; Brown et al., 2008). The alternative common name, mignonette orchid, derives from the superficial similarity between the inflorescences of *Microtis* spp. and that of the dicot mignonette, *Reseda* spp. (Brown et al., 2008). Within Australia, *Microtis* is found in a wide variety of habitats, including swamps, grassland, grassy forests, heathland, mallee shrubland, sclerophyll forest and subalpine meadows (Pridgeon et al., 2001; Brown et al., 2008). Flowering of the species found in Australia occurs from late winter through to early summer, with the inflorescence emerging through a fissure (Figure 2.2) on the hollow terete leaf (Jones, 1988; Bates, 1995; Brown et al., 2008). Seed capsules mature and dehisce rapidly, averaging between two to six weeks depending on the species (Bates, 1984; Pridgeon et al., 2001). In addition, most *Microtis* species also reproduce vegetatively, by forming between one to three daughter tubers each season, depending on environmental conditions.

Figure 2.1 a. Natural distribution of *Microtis*, highlighted by grey areas (adapted from Genera Orchidacearum, Volume 2, 2001). b. Distribution and known localities of *Microtis media* within Western Australia, highlighted in red (https://florabase.dpaw.wa.gov.au/browse/map/10954).
Since the genus *Microtis* was first described by Robert Brown in 1810, there has been much conjecture about the number of species, until a full taxonomic revision was completed in 1984 (Bates, 1984). Difficulties in obtaining fresh material for taxonomic determinations, significant changes to floral shape following drying in numerous species and morphological variability within a species lead to a plethora of newly described species and multiple synonymies. *Microtis media* was originally described by Brown (1810), from a type specimen collected from King George Sound (present day Albany), Western Australia. In his 1984 publication, Bates synonymised *M. media* with *M. unifolia* (Forst.f) Reichb.f., which is distributed throughout Australia, New Zealand, across Polynesia, Indonesia and East Asia. However, in his 1990 revision, Bates reinstated *M. media* as a full species endemic to Western Australia based on the following distinctive morphological observations: presence of irregular, granular excrecescences on the labellum margin, very long basal calli and a very shallow dorsal sepal (Bates, 1990). *Microtis media* was afforded three subspecies, *M. media* subsp. *densiflora*, *M. media* subsp. *quadrata* and *M. media* subsp. *media*, which is the species utilised as the experimental plant in this current study. *Microtis media* subsp. *media* is identified by its yellow-green flowers, shallow dorsal sepal and a longer than broader labellum basal callus (Bates, 1990). *Microtis media* subsp. *media* is endemic to south-west Western Australia and has a widespread distribution ranging from Murchison
River in the state’s north to Israelite Bay in the state’s south-east (Figure 1b), and is found in a multitude of habitats ranging from pristine bushland, swamps and granite outcrops, through to disturbed bushland and domestic garden beds (Brown et al., 2008; FloraBase, 2015). As with other species of the genus, *M. media* also reproduces vegetatively through the formation of daughter tubers.

Throughout its range in Western-Australia (Figure 1b), *M. media* has the ability effectively to colonise habitats ranging from pristine bushland to habitats which are extremely disturbed (Brown et al., 2008). In the latter, it can become an invader of established floral beds in gardens where it may even be regarded as a ‘weed’. In the past decade or so, *M. media* has been observed to occur in large numbers throughout Perth, Western Australia, often in cultivated habitats. This population expansion was especially prevalent following the large scale promotion of water-saving initiatives such as mulching gardens to minimise water loss. It now appears that Kings Park and Botanic Gardens in Perth, which also adopted the practice of mulching horticultural beds to minimise water loss, has inadvertently created novel substrates that were well suited for colonization by *M. media*. After initial attempts to control the spread of the orchid by manual removal proved unsuccessful, park officials have resorted to the use of herbicides in order to control further expansion of *M. media* within the garden (K. Dixon, pers. Comm.).

Further evidence of ‘weediness’ comes from the fact that this orchid can sometimes even be found flowering in roof gutters and drainage channels (K. Dixon, pers comm). Thus *M. media*, perhaps with a few other Western-Australian orchid species (*Pterostylis sanguinea* D.L.Jones and M.A.Clem and *Caladenia latifolia* R.Br), occupies the extreme end of a continuum within terrestrial orchids that passes from those with invasive capabilities, to those that are restricted to a narrow range of habitats. Attempts have been made to classify such distinctive ecological patterns according to plant strategy or C-S-R theory (Grime, 2001), in which plants are seen as having been selected as competitors (C – strategists), stress tolerators (S – strategists) or ruderals (R – strategists). According to the C-S-R theory, plants with the invasive capabilities of *M. media*, based in part on the production of large numbers of freely distributed seeds, would be classified as being ‘ruderals’ or R-strategists. However, other attributes of the life cycle of *M. media*, for example, the considerable longevity of
tubers that enable the plant to occur as a long-lived perennial are characteristic of ‘stress tolerators’ or S-strategists, while the well-defined timing of leaf production is associated with periods of maximum potential productivity, as well as regularity of flowering are features of ‘competitors’ or C-strategists.

Clearly, an orchid such as *M. media* does not fit entirely into any of the established categories of the C-S-R theory. Indeed, its life-cycle demonstrates attributes of all three strategies. The specialised nature of orchid ecological strategies was recognised by Harper (1977), who pointed out that natural selection for very high fecundity might be interpreted as a mechanism that increases the chances of effective contact between orchid seed and the fungal symbiont required to enable germination (Harper, 1977). Seen in this light, the invasive capability of *M. media* may be at least to some extent based upon either a very low specificity in terms of its symbiont requirement, or upon selection in favour of a particularly widely distributed fungal partner. These aspects of its ecology are considered later (see Chapter 3 and 4).
2.1.1 Aims

The success of this ‘invasion’ by the ruderal *M. media* provides both a challenge and an opportunity. The challenge is to understand the eco-physiological basis of the success of *M. media*, and the opportunity is to learn more about the role of its fungal symbionts in the facilitation of its invasive capabilities. Before commencing a study of the contribution of mycorrhizal fungi to the success of *M. media*, some analyses of the attributes that might contribute to its ‘success’ as a ruderal were carried out. These included:

a. Establishing the growing season of *M. media*
   - What is the time-frame required by *M. media* to complete its life cycle?

b. How does seed formation (autogamous or allogamous) occur in *M. media*?

c. Does *M. media* seed differ from other orchid seeds in terms of size and weight?

d. Can *M. media* seeds germinate asymbiotically on water agar?
   - What is the optimal treatment for seed surface sterilization?
   - Are there any effects of light exposure on *M. media* seed germination?
   - To what stage can *M. media* seeds develop asymbiotically when sown on water agar? And if so, is the occurrence unique to *M. media*, or does it occur with other sympatric orchid species?
2.2 Materials and methods

2.2.1 Development of *M. media* from dormant tuber to flowering plant

To determine the life cycle of *M. media*, adult plants (n = 30) from various known habitats (horticultural beds and vagrant plants throughout the living collection) throughout Kings Park were observed weekly from April to October 2012. To determine adult *M. media* shoot and root length, an additional 15 plants of similar size were sampled opportunistically from similar disturbed habitats in late June/early July (after a plateau in shoot growth was observed), washed under a stream of flowing water to remove all soil, scanned and analysed using the WinRHIZO root scanner programme (Regent Instruments, Quebec, Canada).

2.2.2 Seed formation in *M. media*

The extent to which *M. media* was autogamous was examined by conducting pollinator exclusion experiments (Sun, 1997) on vagrant plants found within the Kings Park living collection. Inflorescences (n = 30) were either left un-bagged (n = 15), or were bagged with nylon mesh bags (n = 15, 500 µm mesh size) following inflorescence development in order to exclude any pollinators. Following inflorescence maturation, the total number of flowers per inflorescence was recorded in both the bagged and un-bagged inflorescences. Bagged inflorescences required the brief removal of the enclosing mesh bags to allow for the total number of flowers to be recorded. Seed capsule formation was scored as a measure of successful pollination in both bagged and un-bagged treatments. Mesh bags were kept on until seed capsule dehiscence. Following seed capsule maturation, the total number of successful seed capsules formed per inflorescence was recorded.

In the course of this experiment, both bagged and un-bagged inflorescences were observed on a biweekly basis as part of the maintenance routine. As such, pollination of flowers on the un-bagged inflorescences was recorded whenever observed.
2.2.3 Seed morphometrics

The lengths and widths \((n = 100)\) of field collected seeds were measured every year, following dehiscence, for a total of four years (2011 to 2014). Seeds were viewed using an Olympus SZC16 research stereo microscope (Tokyo, Japan), with length and width measurements carried out with an attached Nikon digital sight DS – L2 imaging controller (Tokyo, Japan). Individual seed weights were calculated from five seed lots, each containing approximately 1000 seeds. Seed lots were weighed on an analytical balance (five decimal places, Shimadzu, AUW120D, Kyoto, Japan).

The ultra-structure of \(M.\ media\) seed was investigated by mounting seeds (approximately 20 seeds per stub, total of ten stubs) on a stub using double sided carbon tape before gold coating with a sputter coater (K550X Sputter coater, Emitech, USA). Coated seeds were then examined for their ultra-structure with a scanning electron microscope (Joel, JCM-6000, Neoscope SEM, Joel, Netherlands).

2.2.4 Investigation of the ability of \(M.\ media\) seed to germinate on water agar

The first aim of the seed germination investigations was to determine if \(M.\ media\) seeds can germinate asymbiotically on a medium, water agar, which lacks supplementary nutrients. Approximately 1000 seeds were heat sealed into individual mesh bags (80 µm pore size), before being immersed in a 100 ml solution of 1 % w/v calcium hypochlorite (supplemented with two drops of Tween 20) and subjected to constant agitation for 15 minutes. This was conducted under sterile conditions within a laminar flow cabinet. Mesh bags were then aseptically removed from the sterilizing solution, rinsed three times (40 ml each) in sterile distilled water, transferred into a sterile petri-dish (containing 10 ml of sterile distilled water), and cut open in order to release the sterilized seeds.

The substrate utilised in these germination experiments was water agar (6 g\(^{-1}\) L agar in 1L of distilled water, sterilized by autoclaving: 15 PSI at 121 °C for 20 minutes). Approximately 20 ml of sterilized molten water agar was dispensed into each petri-dish (90 mm, TechnoPlas, St Marys, South-Australia) and allowed to solidify under sterile conditions before use.
Approximately 80 – 100 seeds were sown onto each petri-dish by pipetting 100 µl of sterile water (containing the sterilized seeds) onto each petri-dish, and spreading the seeds across the agar surface using a sterile glass spreader rod. These petri-dishes (n = 15) were then sealed with Parafilm ‘M’ (Pechiney plastic packaging, Chicago, IL, USA), wrapped up in two layers of aluminium foil to ensure complete darkness, and incubated at 15 °C for four weeks before scoring for germination as per Clements (1998).

2.2.4.1 Optimising duration of seed sterilization, and investigating the effects of light inhibition of M. media seed germination

After establishing the ability of M. media to germinate asymbiotically on water agar, an additional set of experiments, again using water agar, was conducted in order to optimise the process for study of asymbiotic germination.

A range of sterilization periods were investigated to determine the optimal for M. media seed surface sterilization. Seeds were heat-sealed into mesh bags and surface sterilized with 1 % w/v calcium hypochlorite (with two drops of Tween 20). Seeds were exposed to four different sterilization periods (5, 10, 15 and 20 minutes), before being sown onto water agar. A total of ten petri-dishes for each sterilization treatment were sown. Petri-dishes (of each sterilizing treatment) were then incubated under either light (n = 5, 16 hour photoperiod with irradiance provided at a flux density of ~ 30 µmol m⁻² s⁻¹ from high output 20 W cool white fluorescent lamps) or dark (n = 5, achieved by wrapping petri-dishes in two layers of aluminium foil) conditions in order to investigate the effects of light exposure on M. media seed germination. Petri-dishes were incubated at 15 °C for a total of four weeks before scoring.

2.2.4.2 To what stage can M. media seed develop asymbiotically on water agar?

Following the determination of the ability of M. media seed to germinate asymbiotically on water agar, and of the optimal conditions required to introduce M. media seeds into in-vitro culture, a final experiment was conducted to investigate how far M. media seeds could develop asymbiotically while on water agar. Seeds were sterilised for ten minutes (with conditions set out in section 2.2.4), and incubated in light (highlighted in section 2.2.4.1) at 15 °C for a total of twenty weeks before scoring.
of the extent and nature of seedling development. In addition, the following sympatric terrestrial orchid species: *Caladenia latifolia, Caladenia huegelii, Cyanicula gemmata, Diuris magnifica, Ericksonella saccharata, Eriochilus dilatatus* subsp. *dilatatus, Pheladenia deformis, Spiculea ciliata* and *Thelymitra benthamiana* were also introduced into *in-vitro* culture using conditions identical to *M. media*, and incubated under similar conditions for 20 weeks, for use as comparisons.

2.2.5 Data Analysis

Normality testing was conducted using the Kolmogorov-Smirnov Test, and data which conformed to conditions of normality were analysed with a one way Analysis of Variance (ANOVA). When an effect was detected, pairwise comparisons were conducted with Dunnett’s C. Data which did not conform to conditions of normality even after transformations were analysed with non-parametric methods (Kruskal Willis and Mann-Whitney *U* tests). All statistical analyses were performed with SPSS version 17 (IBM, NY, USA).
2.3 Results

2.3.1 Development of *M. media* from dormant tuber to flowering plant in nature

The growth cycle of adult *M. media* plants (dormant tuber – flowering plant – seed capsule dehiscence – dormant tuber) found within Kings Park was completed within 18 to 23 weeks (see Figure 2.3). As with most Western-Australian terrestrial orchids, the re-emergence of dormant *M. media* tubers only occurred after the onset of the first autumn rains, following the orchids’ summer dormancy. In Perth, this period typically occurs from late April to early May. Table 2.1 highlights the increased differences in mean diurnal temperature and rainfall in May (highlighted in bold), as compared to the previous months.

Table 2.1. Monthly climate statistics (1993 – 2015) of the Perth Metro region (weather station location: latitude 31.92° south, longitude 115.87° east). Air temperature is presented in degrees Celsius (°C), as mean minimum and maximum temperatures for the specific month. Mean rainfall for the respective months are presented in millimetres (mm) of rain collected. Information was obtained from the Australian Government Bureau of Meteorology (www.bom.gov.au).

<table>
<thead>
<tr>
<th>Months</th>
<th>Jan</th>
<th>Feb</th>
<th>Mar</th>
<th>Apr</th>
<th>May</th>
<th>Jun</th>
<th>Jul</th>
<th>Aug</th>
<th>Sept</th>
<th>Oct</th>
<th>Nov</th>
<th>Dec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum temperature (°C)</td>
<td>31.2</td>
<td>31.7</td>
<td>29.6</td>
<td>25.9</td>
<td><strong>22.4</strong></td>
<td>19.3</td>
<td>18.4</td>
<td>19.1</td>
<td>20.3</td>
<td>23.3</td>
<td>26.5</td>
<td>29.1</td>
</tr>
<tr>
<td>Minimum temperature (°C)</td>
<td>18.1</td>
<td>18.4</td>
<td>16.6</td>
<td>13.8</td>
<td><strong>10.6</strong></td>
<td>8.5</td>
<td>7.6</td>
<td>8.3</td>
<td>9.6</td>
<td>11.4</td>
<td>14.2</td>
<td>16.4</td>
</tr>
<tr>
<td>Rainfall (mm)</td>
<td>15.4</td>
<td>8.8</td>
<td>20.5</td>
<td>36.5</td>
<td><strong>90.5</strong></td>
<td>127.9</td>
<td>146.7</td>
<td>122.8</td>
<td>89.6</td>
<td>39.5</td>
<td>23.8</td>
<td>9.9</td>
</tr>
</tbody>
</table>

In 2012, *M. media* tuber dormancy was broken on average between one to two weeks following the first major rainfall event (11.6 mm of rain recorded on the 10th of May, 2012, recorded in the Perth metro area) of the season, with shoot and root growth proceeding concurrently. Shoots broke the soil surface within three weeks of the rainfall event, and continued to grow in the ensuing three to five weeks (Figure 2.3a – d). Roots were observed to be growing in an upward direction (Figure 2.3 a, b and c), and the possible biological significance of this observation is discussed in Chapter 7.

This was then followed by a period lacking visible shoot growth (between two to four weeks, indicative of full shoot development), before the emergence of the
inflorescence from a fissure on the hollow shoot (Figure 3e). Of the 15 *M. media* adult plants measured after completing shoot growth, each had a mean shoot length of 25.84 ± 0.94 cm, and an average root length of 5.13 ± 0.26 cm (with an average of 7 roots present on each plant, refer to section 2.2.1 for sampling methods). Concurrent with inflorescence development, the distal formation of droppers (elongated root like structure on which a daughter develops) were observed, with most sampled plants developing between one to three daughter tubers.

2.3.2 Inflorescence development and seed formation of *M. media*

Inflorescences matured rapidly, with floral anthesis occurring within seven to fourteen days of inflorescence emergence (Figure 2.3f), and each inflorescence consisted of an average of 106 ± 8.46 flowers. Flowers matured rapidly (Figure 2.4), and seed capsule formation was often observed within a week of floral anthesis as indicated by the swelling of the ovary. Seed capsule maturation and dehiscence occurred in the subsequent four to six weeks, with inflorescences yielding an average of 99 ± 8.35 seed capsules. Concurrent to seed capsule dehiscence, shoot senescence was also observed, and were indicative of the start of orchid dormancy. By the time the final seed capsules dehisced (Sep to Oct), most plants were already dormant.

2.3.2.1 Seed production by autogamy

Autogamy was observed to occur in *M. media* individuals with bagged (pollinator excluded) inflorescences. This started with the general loosening of the pollinium and subsequent release of pollen onto the stigma below. Following that, the ovary was observed to swell and subsequently developed into a seed capsule (Figure 2.4a). Seed capsules matured rapidly, and dehisced on average in the following three to four weeks. Resulting seeds were not specifically tested for seed viability, but were collected and pooled into the 2012 seed lot. No apparent issues with seed viability were observed as the 2012 seed lot yielded levels of germination and development (data not shown) similar to those of the 2010 and 2011 seed lots (seeds collected from field conditions).

Although mean fruit set was higher in bagged individuals when compared to the un-bagged individuals (Table 2.2), no statistical differences were observed between
treatments. This reduction in seed capsule numbers (un-bagged individuals) was due to herbivory as multiple semi-eaten seed pods were observed to have subsequently formed the pupal case of the herbivore. As pupal cases were no longer inhabited at the time of data collection, identification of the insect herbivore could not be undertaken.

Table 2.2 Autogamous and allogamous fruit set in *M. media* as determined by bagged and un-bagged inflorescences (n=15 inflorescences for each treatment). No statistical differences were observed between the two treatments (MW test, P > 0.05).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean fruit set (%)</th>
<th>Standard error (±)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(total number of seed capsules / total number of flowers)</td>
<td></td>
</tr>
<tr>
<td>Non bagged</td>
<td>93.46</td>
<td>1.64</td>
</tr>
<tr>
<td>Bagged</td>
<td>98.84</td>
<td>0.40</td>
</tr>
</tbody>
</table>
Figure 2.3 Developmental cycle of *M. media*, tracked across 21 weeks after the onset of autumn (May 2012). 

- **a.** Tuber coming out of dormancy as indicated by the beginnings of shoot and root development (week two).
- **b, c, and d.** Subsequent weekly observations of root and shoot growth from tubers of similar size.
- **e.** Emergence of the inflorescence from a fissure on a fully grown shoot (week 12).
- **f.** Floral anthesis occurring on the same inflorescence, in a spiral pattern starting from flowers situated at the base of the inflorescence (weeks 13 to 14). Note the beginnings of seed capsule formation on the lowest flower, as indicated by the swelling of the area directly behind the flower.
- **g.** Dehiscence of seed capsules and dying back of shoot, indicative of the plant entering dormancy (weeks 18-21). Scale bars indicate 1 cm.
Figure 2.4  

a. Development of *M. media* flowers. 

**a1.** Unopened floral bud, 3 days before anthesis. 

**a2.** Newly opened flower on the day of anthesis. 

**a3.** Seed capsule formation seven days after anthesis, as indicated by the swollen ovary. 

b. Floral structure of *M. media*, highlighting the different areas: 

- a. dorsal sepal, 
- b. anther, 
- c. pollinium, 
- d. viscidium, 
- e. stigma, 
- f. petal, 
- g. nectar producing labellum, 
- h. sepal. 

c. Close up of the pollinium, attached to a wooden toothpick by the sticky viscidium. 

Scale bars: Yellow – 3 mm, Green – 1 mm, Red – 500 µm.
2.3.2.2 Seed production mediated by pollinators

Pollination of un-bagged *M. media* inflorescences was observed to be mainly facilitated by workers of the native ant genus, *Iridomyrmex*. The pollinium of *M. media* forms a series of four sheets, each a coherent mass of pollen grains. Each sheet is joined together by the sticky viscidium (Figure 2.4c), and the viscidium forms the point of attachment by which the pollen is removed by the pollinator in one pollen mass. Ants were routinely observed to visit newly opened flowers, and specifically targeted the nectar-containing labellum (Figure 2.5.b.g). Nectar foraging was observed to last between two to five seconds per flower, with ants observed to brush against the adjacent sticky viscidium with their frontal lobes while feeding. This often resulted in the attachment of the sticky viscidium and accompanying pollinium onto the frontal lobes of the feeding ant, followed by the removal of both structures in their entirety when the ant alighted from the flower (Figure 2.5b).

Most ants attempted to remove the pollinium after its attachment by fastidious grooming, but the majority gave up after a few attempts, and proceeded to visit other flowers. As previously highlighted, ants were specifically attracted to the nectar-containing labellum, and whilst feeding on nectar, the ants would indirectly push the attached pollinium against the stigma of the flower they were visiting, thereby facilitating pollination. Although visitation of newly opened flowers was preferred, it was not a mutually exclusive event limited to one ant visit per flower, as many of the newly opened flowers were visited multiple times by multiple ants.

Pollinated seeds were not specifically tested for viability, but were collected and pooled into the 2012 *M. media* seed lot. These seeds were shown to be viable when utilised in subsequent germination experiments as they yielded similar levels of germination to those of the field collected seed lots of 2010 and 2011.
2.3.3 Seed measurements

*Microtis media* seed coats were observed to be loose and papery, with a reticulated surface, and were generally similar to the seed coats of other temperate terrestrial orchids (Arditti & Ghani, 2000). However, subsequent investigations utilising electron microscopy revealed circular openings along the ridges of the seed coat (Figure 2.6).

Seed length and width measurements were undertaken for four different seed batches per season for a total of 4 seasons (2011-2014). No statistical differences were observed in length or width measurements across the different seasons (MW test, P > 0.05), so all measurements were combined to generate a mean seed length of 387.0 ± 3.5 µm, and a mean seed width of 111.0 ± 1.3 µm.
Although measurements of individual seed weights were attempted, instruments sensitive enough to detect the weight of an individual seed could not be sourced. Pooled seed lots containing approximately 1000 seeds had to be utilised and the weight of individual seed interpolated. The approximate mean weight of a single *M. media* seed was determined as 0.826 ± 0.013 µg (826 ± 13 ng).

2.3.4 Asymbiotic *M. media* germination

2.3.4.1 Water agar, 4 week incubation

An initial study was carried out to determine if it was possible to asymbiotically germinate *M. media* on water agar containing no added nutrients. Scoring of germination plates after a four week incubation period (in the dark) showed that 44.92 ± 3.43% of the sown *M. media* seed had developed into non-photosynthetic stage three protocorms (refer to Batty (2001) for specifics of scoring orchid seed germination), with mean lengths of 0.35 ± 0.011 mm, and mean widths of 0.214 ± 0.010 mm. No development beyond stage three was observed by the conclusion of the four week incubation.

2.3.4.2 Sterilization and light treatments

All seeds subjected to the shortest sterilization period (five minute) were infested with either bacterial and/or fungal contaminants, while those exposed to the 20 minute sterilization period were all killed by the treatment (Table 2.3). The ten minute sterilization treatment was determined as the optimal treatment as it resulted in complete seed sterility and subsequently yielded higher seed germination (compared to the 15 minute treatment, Table 2.3). No visible light inhibition was observed, as similar levels of germination were observed in seed kept both in the dark and exposed to light (Table 2.3). As such, all subsequent in-vitro germination of *M. media* seeds were conducted with a ten minute seed sterilization treatment, with sown seeds germinated under a 16 hour photo-period.
Table 2.3 Different *M. media* seed sterilization treatments, and their subsequent seed germination yields. Seeds were sown asymbiotically on water agar, and incubated for four weeks at 15°C, under either light or dark conditions. No statistical differences (MW test, $P > 0.05$) were observed between the light and dark treatments at 10 and 15 minutes sterilization. However, statistical differences (MW test, $P < 0.05$, denoted by different letters) in germination were present within the light and dark treatments, between the 10 and 15 minute sterilization. No germination was observed in the 20 minute treatment. Seeds subjected to the five minute treatment were completely contaminated, either by bacteria or fungi (denoted by -).

<table>
<thead>
<tr>
<th>Sterilizing duration (minutes)</th>
<th>Germination ± SE (%) Light</th>
<th>Germination ± SE (%) Dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>$62.15 \pm 5.56^a$</td>
<td>$63.51 \pm 3.55^a$</td>
</tr>
<tr>
<td>15</td>
<td>$35.62 \pm 5.60^b$</td>
<td>$36.79 \pm 3.22^b$</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

2.3.4.3 Water agar, 20 week incubation

At the end of the 20 week incubation period, protocorms of varying stages (stage one to five) were observed. Numerous green stage five *M. media* ‘protocorms/seedlings’, were observed, indicative of the ability of *M. media* seeds to germinate and develop asymbiotically (to a green protocorm stage) on water agar. These green ‘seedlings’ were extremely small (mean length of $0.35 \pm 0.01$ mm, and width $0.21 \pm 0.01$ mm), deformed and stunted (Figure 2.7), when compared to symbiotically germinated seedlings (see Chapter 3 and 4 for symbiotic seedling pictures and measurements). Investigations into the photosynthetic capacity of these seedlings could not be carried out due to the minute sizes of these seedlings, though seedlings were conspicuously green (Figure 2.7) and may be potentially photosynthetic. Of the nine comparison orchid species, only *Thelymitra benthamiana* developed into stage three protocorms at the end of the 20 week incubation period (Table 2.4). Seeds of the remaining eight species only imbibed (stage one/two), but did not develop further.
Table 2.4. Developmental stages achieved by *M. media* and nine comparison orchid species (n = 10 Petri-dishes per species), 20 weeks after sowing asymbiotically on water agar. **Stage 1:** Imbibing and swelling of seed. **Stage 3:** Formation and growth of multiple trichomes, **Stage 5:** Development and growth of a green leaf. Presence of protocorms of corresponding stages are indicated by +, while the absence is indicated by -.

<table>
<thead>
<tr>
<th>Orchid species</th>
<th>Stage 1</th>
<th>Stage 3</th>
<th>Stage 5</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Microtis media</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Pheladenia deformis</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Cyanicula gemmata</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Diuris magnifica</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Eriochilus dilatatus</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Thelymitra benthamiana</em></td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Caladenia latifolia</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Caladenia huegelii</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Ericksonella saccharata</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Spiculea ciliata</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 2.7 Asymbiotically germinated (water agar) *M. media* stage five protocorms after a 20 week incubation period. Note the highly deformed ‘shoots’ and the greening of otherwise non-green protocorm tissue. Scale bar represents 0.5mm.
2.4 Discussion

2.4.1 Development of M. media from tuber to flowering plant in nature

As highlighted by Brown et al (2008), most of the orchids within Western Australia are geophytic in nature, meaning to say that they survive the long, dry and hot summers by dying back to dormant fleshy underground tubers or rhizomes. Of the orchids found within the South-Western region of Western Australia, the majority of species resprout from dormant tubers in autumn, with dropping temperatures and increased rainfall suggested as triggers which break orchid tuber dormancy (Dixon, 1991; Brown et al., 2008). These orchids would then proceed to complete their life cycles by late spring/early summer (Bates, 1990; Dixon, 1991; Brown et al., 2008; Brown et al., 2014). As such, most geophytic orchids found in the South-Western region of Western Australia share the same seasonal growing cycle (autumn to early summer) and only vary in their flowering times (Dixon, 1991; Brown et al., 2008; Brown et al., 2014).

Results from the current investigation into the growth cycle of M. media in Kings Park, correspond to those of both Dixon (1991) and Brown et al (2008), in showing that dormant tubers re-sprout in early autumn. Re-sprouting was followed by continued shoot expansion, inflorescence and floral development, seed and tuber formation (autumn to mid- winter), with most plants becoming dormant by late winter (see Figure 3 for growth cycle). The current observation that the formation of daughter tubers of M. media takes place distally from the parent plant also corresponds with those of Dixon (1991) who reported Microtis to reproduce vegetatively by developing distal daughter tubers.

The establishment of new M. media colonies within Kings Park often began with a few individuals, with numbers slowly increasing over growing seasons (personal observations from 2012 - 2014). It takes M. media plants (germinated from seed) one growing season to achieve both sexual maturity and to gain the ability to put down multiple daughter tubers (Bates, 1984; Bates, 1990; Dixon, 1991). Under optimal environmental conditions, both flowering (resulting in seed release) and formation of multiple (one to three) daughter tubers occurred in the second growth season. With the onset of the third growing season, the emergence and flowering of multiple M.
media plants were observed. Conditions permitting, seedling recruitment was also observed in the third season. As such, when one extrapolates the colonisation and establishment processes of M. media within novel habitats, one can begin to understand how the abilities to reproduce both vegetatively and by seed could have contributed to the ruderal success of M. media. To further improve our understanding of M. media, levels of clonality within populations of M. media found within Kings Park should be investigated in future studies, which would allow for the determination of how these populations persist, either by vegetative growth, or by seedling recruitment.

2.4.2 Seed production by autogamy

Self-fertilization, or autogamy, has long been regarded as disadvantageous when viewed from a species fitness point of view, as it can result in either inbreeding depression and/or lack of variation in offspring, which limits the ability of the species to adapt to changing environmental conditions (Stebbins, 1974; Wright et al., 2008; Landry & Aubin-Horth, 2013; Krebs et al., 2014). However, autogamy is widespread and common within the plant kingdom, with an estimated 20 to 25% of plant taxa undergoing autogamy (Wells, 1979; Barrett & Eckert, 1990). Within the Orchidaceae, studies have suggested between 5 to 20% of species are able to undergo autogamy as part of their reproductive strategy (Catling, 1983; Catling, 1990; Gale, 2007; Bateman et al., 2015). Autogamy has been suggested as advantageous in the following situations: when environmental conditions such as high rainfall or low temperatures prohibit the effective dispersal of pollen, when populations are founded by one individual (making cross-pollination impossible), and when the homozygous recessive state produces individuals with the highest fitness (Baker, 1955; Baker, 1962; Wells, 1979; Peakall & Beattie, 1991; Peakall & Beattie, 1996). The last situation has been suggested as the driving force behind autogamy in weedy/ruderal species (Baker, 1955; Baker, 1965; Wells, 1979).

As highlighted by Gale (2007), the ability of orchids to utilise autogamy for seed production may translate into the ability to colonize pollinator impoverished/deficient sites, and so potentially expand the natural distribution of the species, which corresponds to what has been observed with the expansion of M. media within
Western Australia. However, autogamous seed production may also result in the accumulation of deleterious conditions such as the loss of genetic diversity, and/or inbreeding depression (Tremblay et al., 2005; Gale, 2007). No apparent issues of seed viability were observed from M. media seeds derived from autogamy, as similar levels (to allogamous seeds) of germination were observed. The facultative ability of M. media to set seed by both autogamy and allogamy highlights the inherent plasticity in seed production of the species, and can be inferred to have contributed to the ruderal expansion of this species within Western Australia.

2.4.3 Seed production mediated by pollinators

Orchids show the greatest floral diversity in the plant kingdom and employ multiple strategies in order to attract pollinators (Chase, 2001). These range from providing food rewards in terms of nectar (occurring in M. media) and/or exudates, to deceptive strategies, such as food or sexual deception (Jeffrey et al., 1970; Stoutamire, 1975; Stoutamire, 1983; Peakall, 1990; Nilsson, 1992; Peakall & Beattie, 1996; Schiestl et al., 1999; Roberts, 2003; Damon & Pérez-Soriano, 2005; Schiestl, 2005; Phillips et al., 2009). Although deception has been proposed as the most common method of attracting pollinators in orchids, with an estimated 10000 orchid species employing some form of deception to attract pollinators (Ackerman, 1986; Peakall & Beattie, 1996), investigations into subsequent fruit set has yielded results which pointed to higher fruit set numbers in nectar producing orchids compared to nectarless orchids (Neilland & Wilcock, 1998).

Orchids are mostly pollinated by insect vectors such as bees, fungal gnats, flies and wasps (Roberts, 2003) but, a few species have evolved to be pollinated by ants (Ackerman, 1986; Peakall et al., 1987; Peakall & Beattie, 1989; Peakall & James, 1989; Peakall & Beattie, 1991). Ants are rarely reported as pollinators (Formicophily) due to a few reasons: inherently small size (limiting pollen load), fastidious and frequent grooming leading to the removal of any attached foreign bodies, relatively smooth exoskeleton limiting the attachment of pollen, limited range (restricting pollen flow) and the production of antibiotic secretions from the metapleural glands which disrupt normal pollen function (Beattie et al., 1984; Beattie et al., 1985; Beattie et al., 1986; Peakall & Beattie, 1989; Damon & Pérez-Soriano, 2005).
Within Australia, only two terrestrial orchids have been recognised as being ant pollinated; *Leporella fimbriata* (Lindl.) and *Microtis parviflora* R.Br, having fulfilled all three conditions set out by Peakall and Beattie (1991) to ascertain ant pollination (Jones, 1975; Peakall et al., 1987; Peakall & Beattie, 1989; Peakall & Beattie, 1991; Peakall & Beattie, 1996). These conditions include: observations confirming the movement of pollen from anther to ant, observations confirming the transfer of pollen from ant to stigma, and observations and/or experiments confirming viability of the resulting seed.

*Microtis media* can tentatively be added as a potential third ant pollinated orchid species within Australia, having met all three conditions: pollinium removal by native *Iridomyrmex* ants, together with the transfer of pollen from the attached pollinium to the stigma of subsequent flowers and production of viable seeds. It is important to point out that these ant pollination events were observed on *M. media* growing in cultivated conditions, and not from plants growing in field conditions. Although numerous *M. media* inflorescences (under field conditions) have been observed to be pollinated by ants (pers. obs.), the random nature of pollination meant that these field observations could not be tabulated. As such, the pollination of *M. media* by other insect vectors cannot be discounted.

### 2.4.4 Seed morphometrics

Orchid seeds, the majority of which are lacking an endosperm and have embryos made up of a cluster undifferentiated cells, are tiny, both in terms of dimensions and weight when compared to seeds of other angiosperms (Arditti, 1967; Rasmussen, 1995; Baskin & Baskin, 2014). As such, most orchids are wind dispersed, and have naturally low establishment and survival rates (Arditti & Ghani, 2000; Chase, 2001). To compensate, orchids have adopted the strategy of producing copious amounts of tiny seeds (with minimal nutrient reserves) in order to maximise offspring dispersal and survival (Arditti & Ghani, 2000; Chase, 2001).

Studies have reported orchid seed lengths to range from 170 µm (*Habenaria obtusata* (Banks ex Pursh) Richards), to 3850 µm (*Epipedium* hybrid), and seed widths to range from 90 µm (*Calypso bulbosa* (L.) Oakes), to 290 µm (*Limodorum abortivum* (L.)Sw) (Clifford & Smith, 1969; Harvais, 1974; Arditti & Ghani, 2000). Similar levels of
variation are observed in orchid seed weights, which range from 0.3 µg in *Schomburgkia undulata* L. to 8 µg in *Gymnadenia* sp R.Br (Koch & Schulz, 1975; Arditti & Ghani, 2000). Although the length (387 ± 3.5 µm) and width (111 ± 1.3 µm) measurements of *M. media* seeds fall well within the reported length and width ranges, the weight of *M. media* seeds are on the lower end of the scale, at 0.826 ± 0.0133 µg.

All of the *M. media* seeds viewed under an electron microscope had obvious circular openings along the ridges of their seed coats (testae), which potentially could facilitate the entry of water into the seed. This is in contrast to other orchid species, most of which have seeds with intact testae, and are highly water repellent due to the presence of a water repellent lipid layer on the testae (Rasmussen, 1995; Arditti & Ghani, 2000).

Difficulty in the entry of water and subsequent imbibing of the seed has been noted as one of the main reasons preventing the successful germination of orchid seeds (Arditti, 1967; Van Waes & Debergh, 1986; Rasmussen, 1995; Mweetwa et al., 2008). This water repellency in seeds can however, be circumvented with exposure to oxidising agents such as sodium or calcium hypochlorite, with seeds imbibing rapidly following such treatments (Arditti, 1982; Hadley, 1982; Van Waes & Debergh, 1986). In the case of *M. media*, the presence of openings along the testae, coupled with the use of calcium hypochlorite (indirectly removing the lipid layer) when introducing seeds into *in-vitro* culture might explain why *M. media* seeds imbibe and develop so rapidly while in culture (see Chapter 3), without any of the lag periods commonly observed with other terrestrial orchid species (Arditti, 1982; Clements et al., 1986; Clements, 1988; Rasmussen, 1995; Arditti & Ghani, 2000). These *in-vitro* observations, can in-turn be utilised to postulate what occurs in nature. Assuming seeds were dispersed in the previous season, the presence of openings along the seed coat would allow for the rapid imbibition of *M. media* seeds following the onset of autumn rains, thus facilitating their subsequent germination and development.
2.4.5 *Microtis media* germination

2.4.5.1 Water agar

Several widespread and abundant terrestrial orchids [*Epipactis gigantea* (Dougl., Ex Hook.), *Goodyera pubescens* (Wild., R.Br.), *Platanthera hyperborea* (Lindl.), *P. obtusata* (Banks., Ex Pursh., Lindl.), *Dactylorhiza purpurella* (T. Stephen and T.A. Stephen), *D. maculata* (L., Soo), *Coeloglossum viride* (Wild., R.Br.), *Gymnadenia conopsea* (L., R.Br.), *G. odoratissima* (L., Rich.), *Anacamptis morio* (L., R.M. Bateman, Pridgeon and M.W. Chase), *A. pyramidalis* (L., Rich.), *Galeola septentrionalis* (Lour.), *Disa bracteata* (SW.), *Diuris magnifica* (D.L.Jones) and *Thelymitra crinita* (Lindl.)] have been reported to germinate asymbiotically and remain viable for a few weeks on water agar (Stoutamire, 1964; Harvais & Hadley, 1967; Harvais, 1974; Stoutamire, 1974; Harvais & Raitsakas, 1975; Smreciu & Currah, 1989; Rasmussen, 1995; Batty *et al.*, 2001; Bonnardeaux *et al.*, 2007). Most of the orchids mentioned above developed asymbiotically to at least stage three protocorms when sown on water agar. As a result, it has been suggested that the nutritional requirements of these seeds are not the limiting factor for initial germination. However, the lack of further development of these protocorms, coupled with subsequent protocorm death also indicates that additional inputs of nutrients are required for any subsequent protocorm development.

Results from the present study, which have shown *M. media* seeds to germinate asymbiotically and develop into tiny and malformed green seedlings on water agar somewhat correspond to the observations of abovementioned studies. These indicate that *M. media* seed might contain sufficient carbon nutrient reserves to allow for initial growth and development, and/or it might even have efficient systems in place for optimal nutrient mobilisation and utilisation (Manning & Van, 1987; Rasmussen, 1995). A further possibility is that the developing protocorms might be able to access small amounts of carbon directly from the agar substrate, since agar utilised in most *in-vitro* plant studies is made up of complex polysaccharides extracted from seaweed (Phillips & Williams, 2009).

Although investigations into the photosynthetic capacities of these seedlings could not be carried out due to size limitations, the atypical development of these seedlings has
led to the conclusion that even if they were capable of photosynthesis, it was insufficient to allow for normal development. Further, the failure of these asymbiotically germinated seedlings to develop normally under asymbiotic conditions strongly suggests that mycorrhizal colonization is a pre-requisite for full development of *M. media* in nature. Subsequent chapters will investigate the functional basis of this apparent dependence upon a fungal symbiont.

2.4.5.2 Sterilization treatments

Sterilization of orchid seed prior to introduction into *in-vitro* culture is usually achieved by the utilisation of oxidising agents such as calcium or sodium hypochlorite (Arditti, 1982). Studies have shown orchid seed germination to increase with increasing sterilizing durations, up to a point, after which germination decreases (Arditti, 1967; Linden, 1980; Van Waes & Debergh, 1986). The oxidising agents act to chemically weather/scarify orchid seed coats, and improve the permeability of the seed coats (and germination) by indirectly removing the water repellent layer (Arditti, 1982; Hadley, 1982; Van Waes & Debergh, 1986). This promotion of seed germination occurs up to a point, after which the seed coats are breached, and the oxidising agents enter the seed (Arditti, 1982; Hadley, 1982; Van Waes & Debergh, 1986; Mweetwa *et al.*, 2008). Following this, seed germination starts to decrease due to the toxic effects of the oxidising agents on the orchid embryo (Arditti, 1982; Hadley, 1982; Van Waes & Debergh, 1986; Mweetwa *et al.*, 2008).

The five minute treatment (which yielded no germination due to contamination) can be inferred as being insufficient for complete seed sterilization, as the seeds themselves were observed to be the source of both fungal and bacterial contaminants. An initial increase and subsequent decrease in germination with increasing sterilizing durations can be observed in *M. media* (Table 3). While the ten minute treatment yielded the best germination, an additional five minutes of exposure almost halved the number of germinants, while an additional ten minutes resulted in no germinants. When combined with the observations of circular openings present on the testae of *M. media* seeds, one can postulate that *M. media* embryos are able to effectively tolerate the oxidative effects of the 1% calcium hypochlorite solution for up to ten minutes,
after which the toxic effects become increasingly pronounced, with complete seed/embryo death occurring after 20 minutes of exposure.

2.4.5.3 Effects of light on *M. media* germination

Germination of seed in some orchid species can be significantly influenced by light exposure (Warcup, 1973; Arditti, 1982; Clements, 1982; Van Waes & Debergh, 1986; Rasmussen, 1990; Perkins *et al.*, 1995; Huynh *et al.*, 2009; Wright *et al.*, 2009; Smith *et al.*, 2010; De Long *et al.*, 2012). In these species, seeds germinate either exclusively in the light (constant illumination and/or normal day-lengths), in complete darkness, or in various light/dark combinations (exposure to the light first before being transferred to complete darkness or vice versa). Although Australian terrestrial orchids are now routinely germinated with an initial period in complete darkness, studies have shown germination to also occur under normal day-length (16 hour photo-periods) conditions (Warcup, 1973; Clements, 1982; Perkins *et al.*, 1995; Huynh *et al.*, 2009; Wright *et al.*, 2009; Smith *et al.*, 2010; De Long *et al.*, 2012). It is thus no surprise that no effects of light inhibition were observed in *M. media*, which yielded similar levels of germination in both normal day-length and complete darkness (Table 3). As such, it can be concluded that as part of their ruderal growth strategy, germination of *M. media* seed in nature is not constrained to any particular soil fraction, and that the species has the ability to germinate both at the soil surface, and at depth.
2.5 Conclusions

With the investigations carried out in this chapter, certain basic eco-physiological features of *M. media* have been established. These include:

- *Microtis media* shares the same growth season with other sympatrically occurring geophytic orchids.
  - Entire life cycle of *M. media* is completed within a short time frame of between 18 to 23 weeks.
- Lack of reproductive constraints within *M. media*
  - Facultative ability of *M. media* to produce seed by both autogamy and allogamy, and vegetative reproduction by the formation of daughter tubers.
  - All of which are adaptations supporting the ruderal habitat of *M. media*
- Seed morphometrics and asymbiotic germination
  - *Microtis media* seeds (length and width) fall within previously reported orchid seed size ranges, although *M. media* seed biomass is very much on the lighter end of the orchid seed weight scale.
  - Circular openings along the seed coats of *M. media* potentially facilitating the ease of water entry into the seed, thereby promoting germination.
  - Lack of photo-inhibition in germinating *M. media* seeds, allowing for germination to occur at both soil surface and at depth.
  - Ability of *M. media* seeds to germinate asymbiotically and persist for extended periods of time on water agar, highlights the non-fastidious requirements of *M. media* seed germination.
  - However, the atypical development of these asymbiotic seedlings also suggests that they would not survive/develop to a more advanced morphological state in the absence of a fungal symbiont in nature.

These aspects of the broader eco-physiology of *M. media* provide support for HYPOTHESIS 1: That multiple aspects of the broader asymbiotic eco-physiology of *M.
media contribute to its success in its natural environment. However, they also demonstrate the absolute requirement for a fungal symbiont to sustain the normal development process. Experiments designed to investigate the symbiotic germination process of this orchid, and to evaluate the patterns of mycorrhizal colonization both in its protocorms and in the roots of field-collected plants will be described in the following chapter.
2.6 References


Chapter 3: The symbiotic germination and development of *M. media*
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3. Abstract

This chapter aimed to characterize the germination/developmental processes of *M. media*, in order to determine if any of these processes contributed to the ruderal success of the species. The rapid symbiotic germination of *M. media* seed, with seeds germinating and developing into stage five and beyond seedlings within four weeks of sowing, has been established. In addition, the rapid development of these *M. media* seedlings have also been characterized, with the entire life cycle of the plant being completed within 20 to 24 weeks. The *in-vitro* flowering of multiple seedlings, and subsequent autogamic production of seeds further supports the autogamic observations of Chapter 2. In addition, the process by which mycorrhizal colonization occurs in *M. media* protocorms (through the suspensor region or through trichomes) and adult roots (through root hairs) has also been described. All of the above observations support the notion that these attributes have contributed to the ruderal success of *M. media*.

3.1 Introduction and aims

Orchid seeds have generally been assumed to lack the ability to germinate and develop independently due to their inherently minute sizes (in terms of size and weight) when compared to seeds of other angiosperms (Arditti & Ghani, 2000; Dearnaley et al., 2012). Since the early studies of Burgeff (1936), it has been considered that associations with mycorrhizal fungi were essential in order to achieve germination (see also Arditti & Ghani, 2000; Dearnaley et al., 2012). The orchid mycorrhizal symbiont replaces the function of a seed endosperm, and promotes seed germination and subsequent development by supplying essential carbohydrates and other required nutrients (Rasmussen, 1995; Arditti & Ghani, 2000; Smith & Read, 2008). It is during this period of symbiotic seed germination and achlorophyllous protocorm development that orchids are assumed to be myco-heterotrophic (Leake, 1994; Rasmussen, 1995).

The successful colonization of orchid embryos by mycorrhizal fungi is usually indicated by the formation of coiled hyphal structures (pelotons) within the developing
Colonization leads to increased meristematic activity and corresponding growth enhancement (Bernard, 1899; Burgeff, 1936; Clements, 1988; Rasmussen, 1995). This growth enhancement (of protocorms) is enabled by fungal provisioning of nutrients acquired from the surrounding environment (Harvais & Hadley, 1967; Alexander & Hadley, 1985).

The orchid/mycorrhizal fungus association is not always stable. Numerous studies have reported instances of initially successful seed colonization and protocorm growth, followed by a switch to virulent pathogenicity by the fungus at a later stage, often resulting in the death of the protocorms (Hadley, 1970; Warcup, 1975; Hadley, 1982; Rasmussen, 1995; Smith & Read, 2008). Very little is known about the factors controlling the balance between parasitism and symbiosis in the orchid mycorrhizal relationship, but as proposed by Smith and Read (2008), the association can be considered a very fine balancing act, where the potential for parasitism by the mycorrhizal fungi is balanced by the defence responses of the orchid. In addition, although most symbiotic orchid germination studies are conducted under relatively constant environmental conditions, germination outcomes can often be highly variable (Warcup, 1973; Clements, 1982; Rasmussen et al., 1990; Rasmussen, 1995; Tan et al., 1998; Smith & Read, 2008; Huynh et al., 2009; De Long et al., 2012), suggesting that even between compatible orchid and fungal partners, symbiosis formation may be physiologically variable.

Within Western Australia, the majority of orchids are geophytic (growing in soil, but seasonally die back to fleshy tubers or rhizomes), with only one true epiphyte (Dendrobium dicuphum F.Muell, found in the Kimberley Region). And of these geophytic orchids, five different patterns of fungal colonization are recognised (Ramsay et al., 1986), as highlighted in Table 3.1. Mycorrhizal colonization of Microtis occurs in the roots (highlighted in bold, Category 4a, Table 3.1)

Although the previous chapter has established the asymbiotic germination and atypical development of M. media seeds into stage five protocorms (small and malformed), the only reason why those asymbiotic structures were classified as stage five protocorms were due to their production of green pigment. Realistically, the sizes of these asymbiotic protocorms were more reminiscent of stage three protocorms. Based on
the atypical development of these asymbiotic protocorms, it was concluded that the presence of a fungal symbiont was likely to be essential for the support of normal protocorm and subsequent seedling growth. Therefore, in this present chapter, the process of symbiotic germination and protocorm development is characterised with *M. media* growing in axenic culture. This analysis is supplemented with a study of the patterns of mycorrhizal colonization within adult *M. media* plants sampled throughout Kings Park.
Table 3.1. Different categories of fungal colonization observed in geophytic West-Australian orchids. Mycorrhizal colonization of *Microtis* belongs to Category 4a (highlighted in bold). Adapted from Ramsay *et al* (1986).

<table>
<thead>
<tr>
<th>Category</th>
<th>Name of fungal colonization</th>
<th>Location of fungal colonization</th>
<th>Orchid species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Stem-tuber colonization</td>
<td>Swollen underground stems</td>
<td>-  <em>Gastrodia, Rhizanthella</em></td>
</tr>
<tr>
<td>2</td>
<td>Underground-stem colonization</td>
<td>Vertical stem between tuber and stem collar</td>
<td>-  <em>Eriochilus, Pterostylis</em></td>
</tr>
<tr>
<td>3</td>
<td>Stem-collor colonization</td>
<td>Swollen region situation at or just below the soil surface, between the underground portion of the tuber shoot and basal subtending leaf</td>
<td>-  <em>Caladenia, Elythranthera, Paracaleana</em></td>
</tr>
<tr>
<td>4</td>
<td>Root colonization</td>
<td>Adventitious roots</td>
<td></td>
</tr>
<tr>
<td>a</td>
<td></td>
<td>Roots radiating laterally from a highly condensed stem immediately atop of tubers</td>
<td>-  <em>Prasophyllum, Microtis, Thelymitra, Diuris, Calochilus, Disa, Spiculaea</em></td>
</tr>
<tr>
<td>b</td>
<td></td>
<td>Roots radiating laterally from the mid-level of a stem stalk</td>
<td>-  <em>Diuris, Lyperanthus, Leporella</em></td>
</tr>
<tr>
<td>c</td>
<td></td>
<td>Similar to 4a, except for the production of ‘pilot’ roots which ascend to the soil surface before bending and growing downwards</td>
<td>-  <em>Prasophyllum, Thelymitra</em></td>
</tr>
<tr>
<td>d</td>
<td></td>
<td>Root tubers which arise adventitiously from an underground rhizome</td>
<td>-  <em>Cryptostylis</em></td>
</tr>
<tr>
<td>5</td>
<td>Root/stem colonization</td>
<td>Combination of categories 4a or 4b with 3, where both collars and adventitious roots are colonized</td>
<td>-  <em>Acianthus, Drakaea, Corybas</em></td>
</tr>
</tbody>
</table>
3.2 Materials and methods

3.2.1 Characterization of the symbiotic germination of *M. media* seeds

In order to characterise the symbiotic germination of *M. media* seeds, a fungal isolate (Kings Park fungal library, Accession number: 2247, identified as *Tulasnella calospora*) previously known to promote *M. media* seed germination was removed from long term storage, and plated onto potato dextrose agar (PDA) (PDA: 6.8 g−1 L potato dextrose powder, 6 g−1 L agar powder, pH 6.8 before autoclaving). The fungal isolate was then incubated at 15 °C for two weeks to allow maximum hyphal growth, and cut into 0.5 mm cubes for use as a fungal inoculum.

The substrate utilised for symbiotic *M. media* seed germination was oatmeal agar (OMA) (2.5 g−1 L of crushed oats and 6 g−1 L agar powder, pH 6.8 before autoclaving). After sterilization, and under sterile conditions (laminar flow cabinet), approximately 20 ml of the molten OMA was dispensed into each Petri-dish, and allowed to solidify before use. Both fungal inoculum (one cube per dish) and sterilized seeds were introduced onto OMA concurrently. *Microtis media* seeds were surface sterilized, sown and incubated as per conditions established in Chapter 2. A total of ten germination plates were sown, and germination was scored on a weekly basis for four weeks (as per Clements 1988). After this initial four week incubation period, seedlings that had reached stage five were subcultured onto fresh OMA on a monthly basis.

3.2.2 Visualization of fungal colonization in stage five *M. media* protocorms

In order to establish the patterns of cellular mycorrhizal colonization within *M. media* protocorms, stage five protocorms of (n = 30) were harvested from germination plates (Section 3.2.1) and cleaned of any attached agar. Protocorms were then fixed in 2.5% glutaraldehyde (in 0.1M Hepes buffer), dehydrated with exposure (30 minutes each) to a graded ethanol (60, 70, 80, 90 and 100 %) series before being embedded using a JB-4 embedding kit (Electron Microscopy Sciences, Pennsylvania, USA) following the manufacturer’s instructions. Polymerised samples were then sectioned (4.5 μm) using glass knives, on a Leica RM 2245 microtome (Nussloch, Germany). Sections were unfurled on water before being mounted on glass slides, and stained with 0.05% w/v
Trypan blue for 5 minutes to highlight areas with mycorrhizal colonization. Slides were viewed with an Olympus BX53-P (Tokyo, Japan) microscope and photographed with an attached Nikon digital sight DS – L2 imaging controller (Tokyo, Japan).

In order to determine the levels of mycorrhizal colonization within these protocorms, protocorms of different stages were cleared for two hours (at room temperature) in an aqueous solution of 10% w/v potassium hydroxide. Cleared protocorms were then neutralised with a few drops of 1% v/v hydrochloric acid before being stained in 0.05% w/v Trypan blue in lacto-glycerol (1:1:1 lactic acid, glycerol and water) for three days, and de-stained in 50% lacto-glycerol for seven days. Entire protocorms were assessed for their levels of fungal colonization and involved the viewing of roots using a Nikon digital sight DS – L2 imaging controller (Tokyo, Japan) attached to an Olympus SZC16 research stereo microscope (Tokyo, Japan).

3.2.3 Visualization of fungal colonization within adult M. media roots

To establish the distribution of fungal colonization in roots of adult M. media plants, twenty adult plants were sampled from bushland within Kings Park in 2014 (early july, after maximum shoot growth was observed). Root systems were cleaned under a stream of flowing water to remove all adhering soil particles before being split into two different treatments: to determine levels of mycorrhizal colonization within these roots, and to determine the cellular patterns of mycorrhizal colonization within these roots (n = 10 per treatment).

In order to determine the levels of mycorrhizal colonization within these root systems, roots were cleared for four hours (at 60 °C) in an aqueous solution of 10% w/v potassium hydroxide. Cleared roots were then neutralised with a few drops of 1% v/v hydrochloric acid before being stained in 0.05% w/v Trypan blue in lacto-glycerol (1:1:1 lactic acid, glycerol and water) for three days, and de-stained in 50% lacto-glycerol for seven days.

Entire root systems of all the ten plants were assessed for their levels of fungal colonization and involved the viewing of roots using a Nikon digital sight DS – L2 imaging controller (Tokyo, Japan) attached to an Olympus SZC16 research stereo microscope (Tokyo, Japan). Fungal colonization of stained root samples was
determined by dividing the field of observation into one mm grids, and visually assessing levels of fungal colonization (% colonization) within each grid.

In order to determine the patterns of cellular colonization within adult *M. media* roots, cleaned roots were fixed, dehydrated, embedded, sectioned, stained and visualised as per conditions set out in Section 3.2.2.
3.3 Results

3.3.1 In-vitro development of symbiotically germinated *M. media* seeds

Development of symbiotically germinated *M. media* seeds were tracked for a total of four weeks, by which time a matted growth of seedlings could be observed on the plates. Germinants of *M. media* exhibited two obvious regions of growth and development: the meristematic region, where the leaf apical meristem develops, and the suspensor region, where most of the mycorrhizal colonization occurs. Seeds imbibed rapidly after sowing, with the majority splitting their testae within 48 hours. This was followed by rapid trichome (simple extensions of epidermal cells) growth and development, with trichomes typically emerging from the lower two-thirds of the protocorms (for example, see Figure 3b.i).

The seeds developed rapidly through the protocorm stages (stages of germination are outlined in Table 3.2), with an average of 20.37 ± 1.59 % of seeds developed to stage three (Figure 3b and 3b.i) within a week of sowing. Stage four protocorms (29.86 ± 6.35%, Figure 3 c) were present by week three, and by week four, protocorms had reached stage five (17.34 ± 2.03%, Figure 3 d - f). Seed germination and protocorm development did not occur uniformly, as protocorms of various developmental stages were observed at all four scoring points (Figure 1). Obvious size differences were present between protocorms of different stages, as highlighted by Figure 3.4.
Protocorms remained translucent in stages one and two, and developed a cream colouration and opacity upon reaching stage three. Concurrent to leaf apical meristem development, stage four protocorms also started producing photosynthetic pigments. This apical meristem subsequently developed into a single green leaf, indicative of a stage five protocorm (see Figure 3.4 d – f). Due to the interwoven trichome development on original germination plates while under in-vitro conditions, sub-culturing had to be carried out within six weeks of sowing (in order to allow for subsequent seedling growth), after which separation of individual seedlings proved challenging.

Batty et al. (2001) established a system for scoring germination stages in terrestrial orchids, adapted from Ramsay et al. (1986) and Clements et al. (1986). These stages were utilised for scoring M. media seed germination (outlined in Table 3.2), with the exception that only M. media protocorms which have developed to stage five and beyond, were considered to have successfully germinated, rather than germination being defined as a stage three protocorm.

Figure 3.1. Development of M. media seeds tracked over four weeks. Colours within each column represent the different protocorm stages observed during that particular week. Error bars denote standard error.
Table 3.2 Stages of seed germination used in this study. [After Batty et al. (2001), Clements et al. (1986), Ramsay et al. (1986)].

<table>
<thead>
<tr>
<th>Germination Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Imbibition of seed, and swelling of embryo (non-green)</td>
</tr>
<tr>
<td>2</td>
<td>Splitting of seed testa (non-green)</td>
</tr>
<tr>
<td>3</td>
<td>Expansion of protocorm and production of multiple trichomes (non-green)</td>
</tr>
<tr>
<td>4</td>
<td>Formation of the leaf apical meristem (non-green)</td>
</tr>
<tr>
<td>5</td>
<td>Leaf elongation (green, germinated)</td>
</tr>
</tbody>
</table>

Although seedling growth continued with subsequent sub-culturing onto fresh OMA, no roots were produced by any of the symbiotically germinated *M. media* seedlings. In addition, no dropper (root-like structures on which the daughter tubers develop) formation was observed. Instead, tubers developed directly at the base of seedlings (at around ≈ 20 weeks after sowing, Figure 3.3.a, i, ii). Following tuber formation, most seedlings entered dormancy. Tubers were dormant for a period of between ten to twelve weeks, after which shoot and root re-growth occurred. The *in-vitro* flowering of *M. media* was also observed in numerous sixteen week old seedlings, just prior to dormancy, with most inflorescences yielding viable seed pods (containing seeds) through autogamy (Figure 3.3b).
Figure 3.3. a. Four month old symbiotically germinated *M. media* seedlings. Note the lack of any root formation in all seedlings, with tubers developing directly at the base of the seedlings. a.i. Close-up of the developing tuber (highlighted by a black circle in a). Note that the surface of the developing tuber is covered in trichomes. a.ii. Identical tuber photographed two weeks later, showing the increase in tuber size. b. *In-vitro* flowering of *M. media* seedlings, five months after sowing. Note the autogamic development of seed pods (black arrows). Scale bars = 2 mm
Figure 3.4 Germination and development of symbiotically germinated *M. media* seeds grown on oatmeal agar. **a.** Imbibing and splitting of seed testa (stage two) a week after sowing. **a.1.** Close-up of a stage two protocorm, showing the entry of mycorrhizal fungi through the suspensor end (green arrow) of the protocorm. **b.** Growth of multiple trichomes (black arrows, stage three) two weeks after sowing. **b.1.** Close-up of a stage three protocorm, highlighting the presence of hyphae within trichomes (indicated by the blue staining). **c.** Development of leaf primordia (white arrows, stage four) three weeks after sowing. **d.** Extension and growth of leaf primordia (white arrows, stage five) four weeks after sowing. **e** and **f.** Development of stage five and beyond seedlings, five and six weeks after sowing. Yellow scale bars represent 200 µm, while green scale bars represent 0.5 mm. All protocorms were cleared and fixed before photography, hence the lack of photosynthetic pigment in pictures **c, d, e** and **f**.
3.3.2 Fungal colonization of protocorms

Contact between seeds and inoculant fungi were observed within three days of sowing. Fungal penetration of seeds resulted in their expansion and coiled fungal structures (pelotons) were observed to have formed within seven days (Figure 3.4 b.1). Depending on protocorm developmental stage, mycorrhizal fungi entered the orchid embryo either through the suspensor end, and/or through trichomes.

If the fungal inoculant encountered a stage two protocorm, colonization occurred only through the suspensor end and spread into the adjacent parenchymal cells [as described by Clements (1988)]. This was then followed by the formation of pelotons within these host cells. Colonization of surrounding and underlying parenchymal cells was then facilitated by trans-cellular hyphae, which penetrated the cell walls of the surrounding cells and developed into pelotons (Figure 3.5 e & f). As trichomes started forming on the surface of protocorms (stage three), the trichomes were colonized by fungal hyphae originating from underlying parenchymal cells. No peloton formation was observed in any of the trichomes, with most hyphae growing through and out of the trichomes, directly into the agar substrate (Figure 3.5 c). In trichomes which developed in protocorm regions yet to be colonized by mycorrhizal fungi, fungal hyphae from the agar substrate were also observed to enter the protocorm through these trichomes, resulting in the fungal colonization of the underlying parenchymal cells. The majority of protocorms in this study were observed to be colonized at stage two. However, if a seed developed into a stage three protocorm before encountering mycorrhizal fungi, fungal colonization occurred through both the suspensor region, and through the trichomes.
Figure 3.5 a and b. Stage 3 and stage 4 protocorms viewed under different forms of illumination (a: above-stage, b: under-stage), highlighting the internal distribution of fungal colonization. c. Multiple hyphae (left of the white star) exiting a single trichome. d. Stained surface of a stage 3 protocorm, showing the presence of multiple hyphae within a single trichome. e and f. Cells directly underneath the trichomes, showing the spread of mycorrhizal infection via trans-cellular hyphae (black arrows). Scale bars represent 1 mm for a, b and c, 200 µm for d and e, and 150 µm for f.

3.3.3 Protocorm cross sections

Similar patterns of cellular colonization were observed across different stages of protocorm development. As such, it was decided that stage five protocorms would be utilised to provide an overall picture of fungal colonization within protocorms (Figure 3.6). The meristematic regions (encompassing roughly a third of a protocorm) of the stage five protocorms were observed to be free of any mycorrhizal colonization (Figure
3.6 region 1, 2, and 3), the remaining two-thirds were observed to be heavily colonized by mycorrhizal fungi (Figure 3.6 region 4 to 8).

In regions free of mycorrhizal colonization, protoxylem structures were first observed in region 4 of the protocorm (Figure 3.6 region 4, highlighted by a black arrow). Moving further along the protocorm away from the suspensor, the leaf apical meristem, and its subsequent development can be observed (Figure 3.6 regions 2 and 3, highlighted by white arrows). The leaf apical meristem at the apex of the stage five protocorm, eventually erupts from the middle of the protocorm as a singular leaf (Figure 3.6 region 1, leaf highlighted by a black double headed arrow).

In regions colonized by mycorrhizal fungi, differences in staining intensity revealed two different states of fungal pelotons (Figure 3.6 regions 5 to 8): pelotons which were still intact, observed as lightly stained purplish lines (Figure 3.6 region 6.a, green arrow), and collapsed pelotons, observed as deeply stained pink clumps (Figure 3.6 region 6.a, blue arrow).

Intact mycorrhizal pelotons were mostly observed in the outer parenchymal region (one to three cell layers underneath the epidermal layer, Figure 3.6, region 7, highlighted by a white double headed arrow), while collapsed pelotons were mostly observed in the inner parenchymal region (region spanning the centre of the protocorm to the outer parenchymal region, Figure 3.6, region 7, highlighted by a red double headed arrow). Cells of the inner parenchymal region containing collapsed fungal pelotons were also routinely re-colonized by fungi from the outer parenchymal layers, as indicated by the presence of both lightly and darkly staining hyphal structures within individual cells (Figure 3.6, 6.a).

The epidermal layer was free of any peloton formation, although fungal hyphae were routinely observed to traverse this layer, either on their way into the protocorm from the agar substrate, or on their way out into the agar substrate from the underlying colonized parenchymal cells, by way of trichomes.
Figure 3.6 A. Transverse sections of a typical stage five protocorm, with figures 1 – 8 representing transverse sections corresponding to positions marked 1 – 8. 1. Leaf tissue prior to eruption from the protocorm, highlighted by the presence of a central ring of cells encircling the vascular bundle.
(black double arrow). 2. Developing leaf primordium indicated by the presence of a small cluster of cells in the middle (white arrow). 3. Protocorm region without any mycorrhizal colonization, corresponding to the start of the photosynthetic region of the protocorm. Note the presence of the leaf primordium (white arrow). 4. Presence of mycorrhizal colonization at the lower left hand side of the protocorm as indicated by the presence of mycorrhizal hyphae (purple lines) within cells. Also note the presence of the protoxylem (black arrow). 5. Middle region of the protocorm with the majority of cells colonised by mycorrhizal fungi. Cells in the middle contain intact active (purplish pink lines) and collapsed (dark purple clumps) hyphae, indicating the re-colonization of those cells by the mycorrhizal fungi. 6. A region similar to that of region 5, with the majority of cells already colonised by mycorrhizal fungi. Note the lack of colonization within the epidermal cells. 6.a. Close up of a cell containing both intact (green arrow) and collapsed hyphae (blue arrow). 7. Lower region of protocorm intensively colonised by the mycorrhizal fungi, with the outer parenchymal region highlighted with a red double headed arrow, and the inner parenchymal region highlighted with a white double headed arrow. 7.a. Close up of the protocorm periphery, highlighting the lack of any fungal colonization within the epidermal cells (empty layer of cells at the edge of the protocorm). 8. Suspensor region of the protocorm, fully colonised by mycorrhizal fungi. Black scale bars represent 200 µm, red scale bar represents 50 µm in picture 6.a and white scale bar represents 100 µm in picture 7.a.

3.3.4 Fungal colonization of adult roots.

One of the asexual reproductive strategies adopted by *M. media* is vegetative reproduction through the formation of daughter tubers. Daughter tubers are formed at the end of droppers: root like structures which grow into the substrate but in which the tip ultimately develops into a daughter tuber some distance away from the mother plant (Dixon, 1991). Of the 20 adult *M. media* sampled from throughout Kings Park, the extent of daughter tuber formation was highly variable, ranging from plants which did not form any daughter tubers, to those which formed between one to three daughter tubers. Clearing and staining of these droppers and their attached tubers revealed the absence of any fungal colonization within these structures, although fungal hyphae were observed to be present on the surface of these structures.

The clearing and staining of adult *M. media* roots (refer to section 3.2.3 for sampling strategy) revealed a patchy distribution of mycorrhizal colonization (Figure 3.7), with an average 56.12 ± 2.59 % of a root length being colonized by mycorrhizal fungi. As in the case of the epidermal layer of protocorms, no fungal pelotons were observed within the epidermal cell layer of adult roots. Fungal hyphae were routinely observed to traverse this epidermal layer as a consequence of colonization process. Fungal pelotons were absent in vascular tissue (Figure 3.7 c.1.i), and in the actively growing
(root meristematic regions. Fungal pelotons were only observed at an average distance of 9.25 ± 0.43 mm distal to the root tip (Figure 3.7.d).

Patterns of cellular colonization of roots were similar to those seen in protocorms, with fungal hyphae entering roots through root hairs, and forming pelotons within the underlying cortical cells (Figure 3.7 a.1, b.1 and d.1). Staining of roots also revealed pelotons in different states: the lighter staining active pelotons, and the intensely staining collapsed pelotons. Cells of the outer cortex (two to three cell layers under the epidermal layer) often contained active pelotons, while cells of the inner cortex (region between the vascular tissue and the outer cortex) often contained collapsed hyphae. Cells containing collapsed pelotons were also observed to be routinely re-colonized by mycorrhizal fungi from surrounding cells (Figure 3.7 c.1.i).
Figure 3.7. Composite picture of a typical *M. media* root, highlighting the patchy distribution of the mycorrhizal colonization (dark blue patches). Note the lack of fungal colonization at the root tip and area immediately behind. A.1. Close-up of area a, showing the entry of hyphae into the root by way of the root hairs, and the subsequent colonization of underlying cortical cells. Colonization of surrounding cells can also be seen, facilitated by trans-cellular hyphae. B.1. Close-up of area b, showing the presence of multiple hyphae within a single root hair and the colonization of cortical cells directly underneath. Note the absence of mycorrhizal hyphae within the surrounding cells. C.1. Transverse section of area c, showing the patchy colonization of the cortical cells. Infected cells are highlighted by the presence of intracellular hyphae (blue lines). C.1.i. Close-up of colonized cortical cells (orange rectangle in C.1), showing the presence of: active hyphae (stars), collapsed hyphae (arrows), nucleus of host cell (circles), and the lack of mycorrhizal colonization within the vascular tissue (double headed arrow). One of the cells containing collapsed pelotons has been re-colonized by mycorrhizal fungi, as indicated by the surrounding of the collapsed peloton by light blue lines. D.1. Close-up of area d, showing the presence of multiple hyphae within a root hair located near the root meristem. Note the movement of fungal hyphae from root hair into the underlying cells, although fungal peloton formation has yet to occur. White scale bars in A.1, B.1 and D.1 represent 1 mm, while black scale bars represent 0.5 cm in 1, 200 µm in C.1, and 100 µm in C.1.i.
3.4 Discussion

3.4.1 Morphological and anatomical development of symbiotically germinated *M. media* seeds

The decision to utilise a *T. calospora* isolate from the King’s Park fungal library was based on previous studies, which have shown the general association of *Microtis* with *Tulasnella* spp. (Milligan & Williams, 1988; Perkins et al., 1995; Bonnardeaux et al., 2007; De Long et al., 2012). The germination and development of *M. media* was rapid (after surface sterilization and concurrent introduction of fungal inoculum), development of seed to stage three protocorms occurring within a week of sowing, and to green stage five protocorms/seedlings within four weeks.

This rapid advancement of symbiotically germinated *M. media* seeds into green seedlings, when compared to the asymbiotic results of Chapter 2, indicates that associations with mycorrhizal fungi are essential for *M. media* seeds to develop normally. In addition, this very early development of autotrophy in *M. media* can be inferred as an adaptation which has significantly contributed to the ruderal expansion of the species.

Results of this investigation suggest that *M. media* is a partial myco-heterotroph (sensu Leake, 1994). Myco-heterotrophs are defined as plants which lack chlorophyll in part of, or all of their lives, being, as a consequence, dependent upon fungi for the provision of carbon and their sustained development (Leake, 1994; Leake & Cameron, 2010). An estimated 20% of all orchid species never produce photosynthetic leaves and are thereby fungus-dependent (full myco-heterotrophs) throughout their lives (Leake, 1994).

Although only about 17% of the sown seeds developed to stage five seedlings at the conclusion of the experiment, it highlights the speed at which germination and development could potentially occur with *M. media* under optimal conditions. Although scoring could not be carried out accurately on germination plates older than four weeks due to the matted growth of seedlings, numbers of stage five seedlings were observed to increase with most protocorms developing beyond stage five in the
subsequent two to three weeks. In general, the percentage of stage five *M. media* seedlings (four weeks after sowing) observed in this study was higher, when compared to two other sympatric and root colonised orchid species, *Disa bracteata* Sw and *Diuris magnifica* D.J.Jones (B. Davis, unpublished data).

Results of this study are in contrast to a previous study, which reported the symbiotic germination (with both Tulasnellaceae and Sebacinaeaceae fungal isolates) of *M. media* to yield less than 20% of stage five seedlings after eight weeks of incubation (De Long *et al.*, 2012). These differences could possibly be attributed to differences in culture media. In DeLong *et al.* (2012) study, the OMA was supplemented with 16 g -1 of sucrose, while no sucrose was added to the OMA utilised in the current study. The decision to omit sucrose from this study was derived from preliminary investigations (data not shown), which showed either sucrose or glucose supplementation to result in the fungal symbiont turning virulent, and parasitising all of the germinating seeds/protocorms. As such, the low percentage of stage five seedlings observed in the De Long *et al* (2012) study might potentially be due to effects of fungal parasitism.

In addition, the scoring systems utilised in the DeLong *et al.* (2012) was adapted from (Batty *et al.*, 2001), and was different to that which was utilised in this study. The rationale behind deeming only stage five and beyond seedlings as successful germinants was to account for the fact that *M. media* seeds have the ability to germinate and develop asymbiotically to a green stage while growing on water agar (see Chapter 2). This was also in accordance with Bonnardeaux *et al* (2007), who suggested that fungal isolates could only be truly deemed efficacious after germination assays have shown the abilities of these isolates to support host orchid seed germination and development to an advanced and autotrophic stage of seedling development.

None of the seedlings produced symbiotically in this study developed any roots. Absence of root formation, was unsurprising as even field collected *M. media* seedlings (in their first season of growth) were routinely observed to lack roots. However, under field conditions, these seedlings (in their first season of growth) would proceed to develop a single dropper, from which a replacement tuber developed. The production of tubers by way of dropper formation was not observed *in vitro* in the current study,
and as such, the formation of tubers at the base of seedlings (observed in this study) should be considered atypical as they are not produced in nature.

It is important to highlight that *M. media* seedlings found in field conditions rarely flower in the season of germination. Although this study has observed the *in-vitro* flowering of symbiotically germinated seedlings in their first season of growth, it should not be inferred as something that routinely occurs in nature. Instead, it should be interpreted as demonstrating the inherent potential of *M. media* seedlings to flower while in their first season of growth, if experiencing optimal growth conditions. This further reinforces the strong R (ruderal) strategy potential of *M. media*, with potentially quick development to reproductive capacity (Grime, 2001).

The *in-vitro* flowering of *M. media* seedlings observed in this study were from seedlings growing symbiotically on OMA containing no additional supplements. These observations are in stark contrast to those of other studies, which have reported the requirement for highly specific and complicated media (including various combinations and concentrations of plant growth regulators and extended plant developmental periods) in order to achieve the *in-vitro* flowering of orchids (Hee *et al.*, 2007; Sim *et al.*, 2007; Tee *et al.*, 2008). The readily achievable *in-vitro* flowering of *M. media* highlights the non-fastidious requirements of this species, and is clearly likely to contribute significantly to its ruderal habit. The subsequent development of viable seed capsules from these inflorescences further reinforces our observation that autogamy occurs in *M. media* (Chapter 2).

3.4.3 Fungal colonization

Patterns of fungal colonization within *M. media* were generally not dissimilar to those described in other orchid species (Clements *et al.*, 1986; Clements, 1988; Peterson & Currah, 1990; Rasmussen, 1990; Richardson *et al.*, 1992; Uetake *et al.*, 1992; Rasmussen, 1995; Peterson *et al.*, 1996). Common to both protocorms and adult roots was the lack of any mycorrhizal colonization within their respective meristematic and vascular regions. This is supported by the commonly accepted notion that plant regions undergoing rapid cellular division and expansion are generally free of any mycorrhizal fungi (Smith & Read, 2008).
3.4.3.1 Protocorm

This study has shown fungal colonization of *M. media* embryos to occur through both the suspensor region, and through trichomes, with no apparent ill effects to either orchid or fungal symbiont. Although mycorrhizal colonization of orchid embryos is generally accepted to occur through the suspensor region, the notion of colonization occurring through trichomes has been a point of contention, despite being reported in numerous publications (Harvais & Hadley, 1967; Arditti, 1982; Clements, 1982; Clements et al., 1986; Clements, 1988; Peterson & Currah, 1990; Rasmussen, 1990; Richardson et al., 1992; Uetake et al., 1992; Rasmussen, 1995; Peterson et al., 1996; Arditti & Ghani, 2000).

Clements (1988) hypothesised that fungal colonization through trichomes was an artificial consequence of the agar substrate, which is usually supplemented with additional soluble carbohydrates such as sucrose or glucose. He thus considered trichome colonization to not be a true reflection of what occurs in nature. It was inferred that the addition of soluble carbohydrates to the germination substrate (indirectly) stimulated seed germination in orchids, resulting in the rapid development of trichomes, and their preferential colonization by mycorrhizal fungi. The results of this study do not support the hypothesis of Clements (1988), since *M. media* seeds have been shown in Chapter 2 to germinate independently and develop multiple trichomes on the most basic and nutrient poor of agar substrates, water agar. In addition, the oatmeal agar substrate utilised in this symbiotic study was not supplemented with any additional soluble carbohydrates. As such, it is unlikely that the trichome formation observed in this study (of *M. media* protocorms) was due to or influenced by soluble carbohydrates within the agar substrate.

Following the initial colonization of underlying parenchymal cells by hyphae entering through either the suspensor region, or through trichomes, colonization of surrounding cells was observed to be facilitated by trans-cellular hyphae. This spread of mycorrhizal colonization by way of trans-cellular hyphae has been previously reported in some orchid species, with the plasmodesmata (microscopic channels traversing plant cell walls) suggested as possible sites from which mycorrhizal fungi moved between cells (Peterson & Currah, 1990; Rasmussen, 1995; Chen et al., 2014).
Investigations into the mycorrhizal colonization of developing stage five protocorms have shown fungal colonization to be relatively well distributed throughout the non-meristematic regions of the protocorm. The presence of active (outer parenchymal region) and collapsed (inner parenchymal region) pelotons present within different protocorm regions also corresponded to the general patterns of fungal colonization observed in other orchid protocorms (Clements et al., 1986; Clements, 1988; Peterson & Currah, 1990; Rasmussen, 1990; Richardson et al., 1992; Uetake et al., 1992; Rasmussen, 1995; Peterson et al., 1996). Although rarely observed, collapsed pelotons were also present within the outer parenchymal regions, implying that although there was a general delineation of protocorm regions based on the state of mycorrhizal hyphae, these regions were not clearly defined. As such, the concept of ‘infection’ and ‘digestion’ cells, and their corresponding roles, as proposed by Rasmussen (1995) should be approached with caution when working with *M. media*.

The observations of this study enable postulations of events occurring in nature. Assuming seeds were all dispersed in the previous season, seed imbibition would occur rapidly with the onset of the autumn rain (Batty et al., 2000; Batty et al., 2006). If mycorrhizal fungi encountered imbibed seeds before the development of trichomes, mycorrhizal colonization would occur through the suspensor region. However, if *M. media* seeds (after imbibing) were present in areas not yet colonized by mycorrhizal fungi, or in areas without compatible mycorrhizal symbionts, the development of multiple trichomes might be an adaptation to explore larger volumes of substrate, hence increasing the chances of encountering compatible mycorrhizal symbionts and encouraging symbiosis formation.

### 3.4.3.2 Roots

At more advanced stages of development, it was established by Ramsay *et al.* (1986) that in nature, it is the roots of *M. media* which become colonized by mycorrhizal fungi. In a study which investigated mycorrhizal fungi within soil collected from West-Australian orchid habitats (within 30 cm of an orchid), the leaf litter and coarse topsoil layers were determined as soil layers which contained the highest fungal activity (Brundrett *et al.*, 2003). Given that the lateral roots of *M. media* normally grow and develop within the top 15 – 20 cm of soil (which encompass the leaf litter and topsoil...
layers, personal observations), the evolution of root colonization can be inferred as an adaptation of *M. media* to exploit this mycorrhizal resource.

The clearing of these roots has shown mycorrhizal colonization to be absent within the vascular and meristematic regions of roots. Fungal colonization was also absent in droppers and daughter tubers, indicating that these structures are not suitable for colonization and play no role in the mycorrhizal-mediated nutrition of the orchid. Tubers are modified root structures used by plants to store nutrients, and in the case of *M. media*, are used by the orchid to survive the harsh Australian summer, and for regrowth in the subsequent season. Mycorrhizal fungi are known to show increased virulence when exposed to freely available carbohydrates, often resulting in plant host mortality (Smith & Read, 2008). As such, the prevention of mycorrhizal colonization from occurring in these nutrient rich structures can be inferred as an adaptation of *M. media* to maximise survivability. By ensuring successful tuber maturation, the chances of surviving summer conditions are significantly increased, which in turn increase the chances of regrowth and flowering occurring in the following season.

Mycorrhizal colonization within adult *M. media* roots was shown to be patchy in distribution. The extent of such colonization within orchid (both epiphytic and terrestrial) roots have previously been reported to vary widely, ranging from little or no colonization, to being almost completely colonized (Hadley & Williamson, 1972; Clements, 1988; Goh *et al.*, 1992; Pereira *et al.*, 2005; Suárez *et al.*, 2006; Pereira *et al.*, 2014). Specifically, the pattern of fungal colonization observed within *M. media* was highly reminiscent of what was reported to occur within a few Malaysian terrestrial orchids (Hadley & Williamson, 1972).

Observations into the beginnings of fungal colonization in roots have shown that an initial infection of a single root hair can result in the colonization of multiple underlying cortical cells. This infection then proceeds to expand into surrounding cells (outer cortical layer), and into the underlying cells (inner cortical layer) by way of transcellular hyphae. Thus, each mycorrhizal ‘patch’ can therefore be inferred to arise from a single infection point. Based on observations from Bonnardeaux *et al* (2007) and De Long *et al* (2012), we can therefore hypothesize that each *M. media* root could potentially be colonized by multiple *Tulasnella* individuals. The colonization of orchid
roots either by multiple individuals of the same fungal species, or by multiple fungal species has previously been reported (Kristiansen et al., 2001; McCormick et al., 2004; Bonnardeaux et al., 2007; De Long et al., 2012; Kohout et al., 2013; Xing et al., 2015).

Therefore, the possibility of *M. media* roots being colonised by multiple fungal genomes cannot be discounted. Such an ability to associate with a number of different symbiotic genotypes may translate into the ability of *M. media* to access different nutritional sources by way of its mycorrhizal partner. The issue of genetic diversity of *M. media* colonists is considered further in the following chapter.
3.5 Conclusions

The symbiotic germination and development of *M. media* seeds has been characterized and the process by which mycorrhizal colonization occurs in *M. media* (protocorms and adult roots) has been described. Specific observations include:

- Association with an appropriate mycorrhizal symbiont results in the rapid growth and development of *M. media* seeds.
  - Seed germination and subsequent development into stage five and beyond protocorms/seedlings was achieved within four weeks of sowing.
  - In accordance with observations made upon field-grown seedlings during their first year of development, no symbiotically grown seedlings produced roots under laboratory conditions.
- The entire life-cycle of *M. media* (including flowering) can be completed under *in-vitro* conditions (without any additional nutritional supplements) within 20 – 24 weeks of sowing.
- All of the above observations suggest that mycorrhizal associations are essential to allow for normal *M. media* development.

- Similarities in the fungal colonization of *M. media* protocorm and adult root tissue.
  - Fungal colonization of developing protocorms occurred either through the suspensor end of stage two protocorms, or from both the suspensor end and through trichomes in stage three protocorms, while the colonization of adult *M. media* roots only occurred through root hairs.
  - Trans-cellular hyphae were responsible for the spreading of mycorrhizal colonization to neighbouring cells in both protocorms and adult roots.
  - No mycorrhizal colonization was observed in the meristematic and vascular regions of both protocorms and adults roots.
- Small physiological differences of *in-vitro* grown seedlings compared to those produced under field conditions.
• Atypical tuber formation occurring directly at the base of in-vitro germinated seedlings, as opposed to the formation of tubers at the distal ends of droppers, as observed in field collected seedlings.
• Flowering of in-vitro germinated seedlings in their first season, as opposed to the second season observed in field grown seedlings.

All of these results have highlighted the need for mycorrhizal associations to support the normal development of *M. media*. They therefore support HYPOTHESIS 2: That aspects of the symbiotic germination process will play a key role in determining the ability of *M. media* to establish in its natural environments. Thus, the speed at which *M. media* seeds germinated and developed in-vitro, when inoculated with an appropriate mycorrhizal symbiont can be inferred as an adaptation which will support the ruderal habit of the species. As this chapter only investigated the symbiotic capabilities of one fungal isolate, the next chapter chapter aims to isolate and identify fungal symbionts from adult *M. media* plants collected throughout Kings Park, and to characterise their abilities to aid in seed germination and subsequent seedling development.
3.6 References


Chapter 4: The taxonomy and some functional attributes of the fungal symbionts forming mycorrhizas with *M. media*
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4. Abstract

Given its ruderal success, the next step in the understanding of *M. media* was to determine its fungal partners/symbionts, in order to determine the fungal specificity of *M. media*. Investigations into the identities and functional attributes of mycorrhizal symbionts associating with ruderal populations of *M. media* have allowed for the establishment of a few key points. The majority of mycorrhizal symbionts were identified as *Tulasnella* species, and all had varying degrees of effectiveness in promoting *M. media* seed germination, and subsequent protocorm/seedling growth. In addition, preliminary baseline values of mycorrhizal length and surface areas are also presented, and highlight the importance of how associations with mycorrhizal fungi potentially contribute to the nutrition of developing *M. media* protocorms. Results of this chapter suggest that the ruderal success of *M. media* might partially be due to its association with the cosmopolitan fungal genus *Tulasnellaceae*.

4.1 Introduction

Most orchid mycorrhizal fungi have generally been considered to belong to the polyphyletic form genus Rhizoctonia within the Basidiomycota (Rasmussen, 2002; Suárez et al., 2006; Bonnardeaux et al., 2007; Brown et al., 2008; Smith & Read, 2008; Dearnaley et al., 2012; Pereira et al., 2014). Members of the mycorhiza-forming genus Rhizoctonia comprise a disparate group of distantly related fungal clades that fall into three different families namely Ceratobasidiaceae, Tulasnellaceae and Sebacinaceae (Rasmussen, 2002; Taylor et al., 2002; Smith & Read, 2008; Dearnaley et al., 2012). Taylor et al (2002) emphasised the large phylogenetic distances between the three basidiomycetous clades. Some ascomycete fungi have also been reported to form symbiotic associations with both fully and partially myco-heterotrophic orchids, especially when the partial myco-heterotrophic orchids are growing under conditions of low irradiance (Bidartondo et al., 2004; Selosse et al., 2004; Ogura-Tsujita & Yukawa, 2008; Ogura-Tsujita et al., 2009; Roy et al., 2009; Dearnaley et al., 2012).

Identification of orchid mycorrhizal fungi has been historically difficult, with few morphological differences among the *Rhizoctonia* and the rarity of observing sexual
sporulation necessary to identify teleomorphic genera (Currah et al., 1997). The application of molecular methods to orchid mycorrhizal fungal identification has provided the means by which to investigate specificity, ecological interactions and phylogenetic relationships between both orchids and fungi (Taylor et al., 2003; McCormick et al., 2004; Otero et al., 2007).

Studies on orchid-fungal specificity have also revealed that orchids grown under in-vitro conditions can associate with a greater diversity of fungal partners than is typically observed in situ (Masuhara & Katsuya, 1994; Perkins et al., 1995; Bonnardeaux et al., 2007). Investigations into the mycorrhizal symbionts of Australian terrestrial orchids have shown the same general association with fungi from the form genus Rhizoctonia (Warcup, 1973; Warcup, 1981; Clements, 1982; Dixon, 1991; Bonnardeaux et al., 2007; Huynh et al., 2009; Wright et al., 2009; Smith et al., 2010; Swarts et al., 2010; Phillips et al., 2011; De Long et al., 2012; McQualter et al., 2013).

As a genus, Microtis has been found to predominantly associate with Tulasnella, although occasional associations with Sebacina have also been reported.

In one of the first studies which investigated mycorrhizal symbionts associated with Microtis R.Br, Warcup (1981) reported the association of Microtis with Sebacina vermifera Oberwinker, and Tulasnella calospora (Boudier) Juel. In the present study, the name Sebacina vermifera (sensu Warcup 1981) is retained despite the fact that the taxonomic position of this fungus is in the process of being revised (Weiss et al., New Phytologist in press). According to this revision, it is likely that S. vermifera will be transferred into the genus Serendipita. Warcup's Sebacina isolates are awaiting full description and taxonomic re-assignment and the likelihood is that all will be re-classified as species within Serendipita (M. Weiss 2015 – personal communication).

The observation that Microtis associates with Sebacina and Tulasnella spp. was further reiterated in a later study. In their analysis of the mycorrhizal symbionts of Microtis species growing in New South Wales, Milligan and Williams (1988) largely confirmed the observation of Warcup (1981), that the normal fungal associates of two Microtis species, Microtis unifolia (Forst.f) Reichb.f. (≡ Microtis media R.Br) and Microtis parviflora R.Br (≡ Microtis eremaea Bates) were Sebacina spp. and Tulasnella spp. Of the 27 fungal isolates obtained by Milligan and Williams (1988), 26 were fast growing
bi-nucleate fungi believed to be *T. calospora*, while the one remaining isolate was of a slow growing multi-nucleate fungus (isolated only once from a single plant) believed to be *S. vermifera* (Milligan & Williams, 1988). Based on these results, the authors concluded that *T. calospora* was the normal mycorrhizal associate of *Microtis*, and that although associations with *S. vermifera* were possible, they rarely occurred. However, based upon the observation that *S. vermifera* was able to stimulate symbiotic development of *Microtis* spp. *in-vitro*, Milligan and Williams (1988) hypothesized that this fungus may play a role in the development of *Microtis* spp. in nature.

In 1995, the mycorrhizal symbionts associated with *M. parviflora* (≈ *M. eremaea*) were examined in detail (Perkins *et al.*, 1995). Results from this (study) corresponded to those of Warcup (1981) and Milligan and Williams (1988), in that the authors isolated only two fungi: *Epulorhiza repens* (Bernard) Moore (≈ *Tulasnella calospora*) and *Epulorhiza* sp. (≈ *Tulasnella* sp.), from protocorms and roots of *M. parviflora* collected from the field. In the laboratory, these fungi and three others, including some saprophytic bi-nucleate ‘Rhizoctonia’s’ belonging to the genus *Ceratorhiza* (≈ *Ceratobasidium*), were also observed to initiate germination of *M. parviflora* seed.

While the ability of *Ceratobasidium* sp. to initiate *M. media* seed germination was reported by Bonnardeaux *et al* (2007), it was listed by the authors as a ‘partially compatible symbioses’, as opposed to a ‘fully compatible symbiosis’, as the *Ceratobasidium* germinated seedlings were subsequently observed to abort. The inability of *Ceratobasidium* to support germination of *M. media* seeds was further reported by De Long *et al* (2012). Although De Long *et al* (2012) managed to isolate *Ceratobasidium* sp. from adult *M. media*, subsequent investigations into the abilities of these fungal isolates to promote *M. media* seed germination revealed that none of these isolates were able to initiate germination.
4.1.1 Aims

Building on the background information provided in the previous section, and on the conditions established in Chapter 3 (symbiotic germination of *M. media*), this chapter aimed to investigate the mycorrhizal symbionts associated with *M. media*. Specifically the aim was to:

- Establish the molecular identities of mycorrhizal fungi associating with ruderal populations of *M. media* growing within Kings Park.
  - Does *M. media* show high fidelity to a particular genus of fungi? Or does it associate with a wide range of fungi?
- Establish the abilities of these fungal isolates to promote *M. media* seed germination and subsequent growth.
  - Are there any differences in the abilities of different fungal isolates to promote *M. media* seed germination?
  - Are there size differences in seedlings germinated with the different fungal isolates?
- Establish baseline length and surface area measurements for both mycorrhizal fungi and their corresponding *M. media* protocorms
  - To determine if there are any differences (in length and surface area) between different fungal isolates
  - To determine if there are any differences (in length and surface area) between protocorms germinated with different fungal isolates.
4.2 Materials and methods

4.2.1 Fungal isolations

In order to characterize the mycorrhizal fungi associated with ruderal populations of *M. media*, adult plants (*n* = 50) of the orchid were sampled opportunistically during early winter (mid-June, after achieving maximum shoot growth) in 2013 from a range of disturbed habitats found within Kings Park. These included vagrant plants appearing within the pots of other plant species within the Kings Park living collection (*n* = 15), horticultural beds (*n* = 20) and disturbed bushland (*n* = 15).

Isolations of mycorrhizal fungi from individual pelotons were carried out using a modified protocol of Smith *et al* (2010). Root sections (~3cm) were washed under tap water for ten minutes to remove any visible soil particles. They were then subjected to constant gentle agitation in sterile distilled water for five minutes (repeated three times per root section). Root sections were then transferred onto a sterile petri dish containing approximately 1ml sterile distilled water, and the root tissue was scraped with a sterile scalpel blade to liberate individual fungal pelotons. Pelotons (suspended in water) were picked up with a sterile glass Pasteur pipette, and passed through four consecutive rinses in sterile water before being transferred onto fungal isolation media [FIM; 0.3 g\(^{-1}\)L sodium nitrate, 0.2 g\(^{-1}\)L potassium dihydrogenorthophosphate, 0.1 g\(^{-1}\)L magnesium sulphate, 0.1 g\(^{-1}\)L potassium chloride, 0.1 g\(^{-1}\)L yeast extract, 2.5 g\(^{-1}\)L sucrose, 9 g\(^{-1}\)L agar, pH 6.8 before autoclaving, 10 mL of streptomycin sulphate stock (0.14 g of streptomycin salt in 70 ml of sterile distilled water) solution added after autoclaving].

These inoculated dishes were incubated at 15 °C for a minimum of three days before any subculturing. Subculturing involved the removal of a single hyphal tip from the actively growing edges of developing fungal colonies and transferring them to fresh FIM plates. This process was carried out continuously over 40 days, until a culture free of contaminants was obtained. Individual fungal isolates were subsequently plated onto potato dextrose agar (PDA; 6.8 g\(^{-1}\)L potato dextrose powder, 6 g\(^{-1}\)L agar, pH 6.8 before autoclaving).
Fungal isolates were screened throughout the subculturing process. Any fast-growing or sporulating isolates were discarded. Isolates with the following characteristics were retained: hyphae branching at right angles, presence of monilioid cells, hyphae lacking in clamp connections and/or formation of hyphal coils on the agar surface (Currah et al., 1997; Garcia et al., 2006).

A total of 22 fungal isolates were obtained, and all were used in the subsequent experiments: molecular identification (see Section 4.2.2) and symbiotic germination (see Section 4.2.3). All 22 fungal isolates were also placed in long term storage, which involved suspending cubes of actively growing isolates (on PDA) in sterile distilled water at 5 °C.

4.2.2 Molecular identification of fungal isolates

Molecular identification of all the 22 fungal isolates was carried out. It involved the growing of all isolates on PDA for three weeks before DNA extractions were carried out as per Swarts et al (2010). Amplification of genomic DNA by polymerase chain reaction (PCR) was achieved using two fungal primer pairs: ITS1-F (5’-CTT GGT CAT TTA GAG GAA GTA A-3’) & ITS4 (5’-TCC TCC GCT TAT TGA TAT GC-3’), and ITS1-F (5’-CTT GGT CAT TTA GAG GAA GTA A-3’) & ITS4-Tul (5’-TCC GTA GGT GAA CCT GCG G - 3’) (Taylor & Bruns, 1999; Taylor & McCormick, 2008; Phillips et al., 2011). Each PCR reaction contained 2µl of fungal DNA extract, 5µl of 5x polymerisation buffer (Fisher Biotech, Western Australia), 3µl of 25nM MgCl, 6.8µl of sterile H2O, 4µl each of the forward and reverse primers (3.2µM) and 0.2µL of TAQ polymerase.

PCR reactions consisted of an initial denaturing at 95 °C for 2 minutes, followed by 36 cycles of: denaturation at 95 °C for 1 minute, annealing at 50 °C for 2 minutes and extension at 72 °C for 1 minute. The cycles were terminated with a final extension at 72 °C for 8 minutes. The products were run on a 1% agarose gel and viewed under UV illumination to confirm genomic amplification. PCR products were sent to AGRF (Australian Genome Research Facility Ltd, Perth, Western Australia) for sequencing. Sequences were manually edited and aligned with Codon code Aligner (v 4.2.3), before performing a BLAST search on the NCBI database (http://blast.ncbi.nlm.nih.gov) to identify the sequences.
4.2.3 Abilities of fungal isolates to promote *M. media* germination and subsequent growth

The ability of all 22 fungal isolates to promote seed germination and protocorm growth were investigated with symbiotic germination studies (see Chapter 3 for basic methodologies). This involved the concurrent introduction of surface sterilized *M. media* seeds (approximately 80 – 100 seeds) and a fungal isolate onto oatmeal agar (OMA). A total of ten germination plates were sown for each fungal isolate and incubated under light conditions (see Chapter 2 for specifics) for four weeks before scoring for seed germination as per conditions established in Chapter 3. To determine mean protocorm length and width, five protocorms from each germination plate (total of 50 protocorms) were measured with a Nikon digital sight DS – L2 imaging controller (Tokyo, Japan) attached to an Olympus SZC16 research stereo microscope (Tokyo, Japan).

4.2.4 Length and surface area measurements of fungal isolates and corresponding protocorms

To investigate fungal symbiont/orchid protocorm lengths and surface area, seeds were germinated symbiotically using three fungal isolates (2.4.1, S.A.1.1 from this study and 7.1.1 (*Tulasnella* sp.) from the Kings Park fungal library). There were five germination plates per fungal isolate, and these were incubated in the dark (by wrapping dishes in two layers of aluminium foil) at 15 °C for three weeks to generate non-green stage four seedlings. Stage four non-photosynthetic seedlings were utilised in this study to avoid any carry over effects of photosynthesis on mycorrhizal growth, as indicated by the movement of carbon fixed by the orchid *Goodyera repens* through photosynthesis to mycorrhizal symbiont in (Cameron *et al.*, 2008).

Individual stage four protocorms (n = 50 per fungal isolate) of similar size were removed from the germination plates and transferred onto water agar plates. These were incubated in the dark at 15 °C for seven days to allow fungal growth to spread across the entire plate. After this, protocorms were removed and analysed (for length and surface area) using the WinRHIZO root scanner programme (Regent Instruments, Quebec, Canada).
To determine fungal length and surface area, a 0.5cm cube of agar (~ 2cm away from the protocorm, n = 15 per fungal isolate) was removed and placed in an Eppendorf tube containing 500µL of sterile water, macerated using the tip of a scalpel blade and incubated in a 65 °C water bath for 30 minutes in order to liquefy the agar. Two drops of Trypan Blue stain were then added to the tube, which was incubated for a further three minutes before removal of supernatant under vacuum filtration. Hyphal lengths of stained hyphae trapped on the filter membrane (Whatman’s polyamide membrane circle, NL16) were then quantified (on a per petri-dish basis) according to a modified grid line intersect method, originally developed for estimating root lengths (Tennant, 1975).

4.2.5 Data Analysis

Data were checked for conditions of normality and homogeneity of variance using the Kolmogorov – Smirnov Test and Levene’s Test, respectively. For data that conformed to the conditions, one way Analysis of Variance (ANOVA) was used to analyse for the effect of the factor on the variable. When an effect was detected, pairwise comparisons were performed with Dunnett’s C. For data that did not conform to the conditions even after transformations, non-parametric methods (Kruskal Willis and Mann-Whitney U tests) were used. All statistical analyses were performed with SPSS version 18 IIBM, NY, USA).
4.3 Results

4.3.1 Fungal isolation and molecular identification

A total of 22 separate fungal isolates were obtained from the 50 adult *M. media* plants sampled from disturbed environments throughout Kings Park (See section 4.2.1 for details). These consisted of 6 isolates from plants sampled from horticultural beds, 11 isolates from plants sampled from disturbed bushland, and 5 isolates from vagrant plants occurring within the Kings Park living collection (Table 4.1). Of these 22 isolates, 18 were successfully identified (Table 4.1) as belonging to the Tulasnellaceae. Although most were only identified to the genus level (*Tulasnella*), two isolates (2.4.1 and S.A.1.1) were successfully identified as *Tulasnella calospora*.

Although genomic DNA of the four un-identified isolates was successfully extracted (presence of bands when genomic DNA extracts were run on a 1% agarose gel, and spectrophotometer readings indicating the presence of DNA), subsequent amplification and sequencing attempts were unsuccessful.
Table 4.1 Putative identities of 22 fungal isolates investigated, with the corresponding primers utilised (+: PCR amplification of genomic extract, - : no PCR amplification of genomic extract). Origins of fungal isolates, together with percentage homology, accession number (on Genebank) and their respective publications are also presented. Isolates unsuccessfully identified are denoted by blank rows. HB: Horticultural beds, DB: Disturbed bushland, VP: Vagrant plants from the Kings Park living collection.

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<th>Primer combination: ITS1-F and ITS4-Tul</th>
<th>BLAST homology (%)</th>
<th>Closest relative</th>
<th>Accession number</th>
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4.3.2 Symbiotic germination of *M. media*

All 22 fungal isolates were capable of stimulating germination and growth of *M. media* (Figure 4.1), with mean germination levels varying widely, ranging from 14.68 ± 4.55% for fungal isolate 16.1.1, to 54.31 ± 4.17% for fungal isolate 2.4.1. Fungal isolate 2.4.1 was observed to be the fungal isolate which yielded the best *M. media* germination (Figure 4.2). A common feature observed across this investigation was that germination was heterogeneous, indicated by presence protocorms of various developmental stages at the time of scoring. Seeds sown on the control substrate (water agar) only developed to stage three protocorms at the conclusion of the experiment, and as such, were not considered to have germinated (data not shown, see Chapter 3 for seed scoring conditions).

**Figure 4.1** Mean germination percentage (comparing the number of protocorms which developed to stage five to the number of seeds sown) of the different fungal isolates investigated. Data of the control (water agar) treatment is not presented due to lack of successful germination. Fungal isolates with * above them are significantly different to the best performing isolate, 2.4.1 (P < 0.00238). Error bars denote standard error.
Length and width measurements of protocorms germinated with the 22 fungal isolates were highly variable (Figure 4.3 a and b), with protocorm lengths ranging from $1.64 \pm 0.32$ mm for protocorms germinated with isolate 4.1.1, to $5.4 \pm 0.46$ mm for protocorms germinated with isolate S.A.1.1. Protocorm widths ranged from $0.68 \pm 0.05$ mm for protocorms germinated with fungal isolate 4.1.1 to $0.96 \pm 0.06$ mm for protocorms germinated with fungal isolate 10.11. Although seeds sown on water agar (controls) were not considered as germinated (only developed to stage three protocorms), their length ($0.35 \pm 0.01$ cm) and width ($0.21 \pm 0.01$ cm) measurements are included in Figure 4.3 a and b, to highlight the obvious size differences between asymbiotic and symbiotic seedlings.
Figure 4.3 Mean M. media protocorm lengths (a) and widths (b) four weeks after sowing, germinated with different fungal isolates, compared to the control (water agar). Columns with * above them are significantly different to isolate 2.4.1 (P < 0.00238). Error bars denote standard error.
4.3.3 Length and surface area measurements of fungal isolates and corresponding protocorms.

Baseline values of lengths and surface areas of mycorrhizal fungi and the protocorms with which they associated were established. Fungal measurements (hyphal lengths and surface area calculated on a per petri-dish basis) and protocorm measurements (lengths and surface area) are presented in Table 4.2. A larger variation was observed within fungal data (statistically significant differences in hyphal length and surface area between different isolates) compared to protocorm data (no statistically significant differences).

Table 4.2 Mean length and surface area of hyphae of three mycorrhizal isolates (calculated on a per petri-dish basis) and their corresponding protocorms. Statistical differences in hyphal length and surface area between different fungal isolates are denoted by different letters after correction for multiple pair-wise comparisons with the Bonferroni method ($P < 0.0167$). No statistical differences were detected in protocorm lengths and surface areas.

<table>
<thead>
<tr>
<th>Fungal isolate</th>
<th>Hyphae</th>
<th>Protocorm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length $\pm$ SE (cm)</td>
<td>Surface area $\pm$ SE (cm$^2$)</td>
</tr>
<tr>
<td>2.4.1</td>
<td>232.81 $\pm$ 8.21$^b$</td>
<td>0.29 $\pm$ 0.01$^b$</td>
</tr>
<tr>
<td>7.1.1</td>
<td>272.08 $\pm$ 9.39$^a$</td>
<td>0.25 $\pm$ 0.01$^a$</td>
</tr>
<tr>
<td>SA.1.1</td>
<td>191.82 $\pm$ 4.80$^a$</td>
<td>0.15 $\pm$ 0.00$^a$</td>
</tr>
</tbody>
</table>
4.4 Discussion

4.4.1 Molecular identification of fungal isolates

This study did not investigate the fungal symbionts of *M. media* originating throughout its distribution (Figure 2.1) as that has already been undertaken in an earlier study (De Long *et al.*, 2012), but focussed on *M. media* found within Kings Park, which encompasses both the natural habitat of *M. media*, and of novel habitats that are being actively colonised by *M. media*. This was carried out in order to determine if there were any mycorrhizal species present which were more functionally capable in promoting *M. media* seed germination and/or protocorm growth, which would then be utilised in the subsequent nutritional studies.

The observations reported here confirm that the most commonly occurring mycorrhizal associates of *M. media* are *Tulasnella* spp. Members of the Tulasnellaceae are soil fungi with a cosmopolitan distribution, and have been reported to exist as both saprophytes and plant symbionts (McCormick *et al.*, 2004; Garcia *et al.*, 2006; Suárez *et al.*, 2006; Smith & Read, 2008; McCormick *et al.*, 2012; Ogura-Tsujita *et al.*, 2012; Tan *et al.*, 2014). Numerous studies, encompassing both epiphytic and terrestrial orchids, have reported *Tulasnella* spp. as mycorrhizal symbionts (Hadley, 1970; Warcup, 1981; Milligan & Williams, 1988; Suárez *et al.*, 2006; Bonnardeaux *et al.*, 2007; Smith *et al.*, 2010; Phillips *et al.*, 2011; De Long *et al.*, 2012; McCormick *et al.*, 2012; Nurfadilah *et al.*, 2013; Pereira *et al.*, 2014; Tan *et al.*, 2014).

Although this study attempted to utilise general fungal primers (ITS1-F and ITS4), previously shown to amplify genomic DNA of orchid mycorrhizal fungi (Shefferson *et al.*, 2005; Taylor & McCormick, 2008; Swarts *et al.*, 2010), little amplification was achieved. Amplification of fungal genomic DNA was only achieved when *Tulasnella* specific primers (ITS1-F and ITS4-Tul) were used; with 18 out of the 22 fungal isolates being successfully identified (Table 4.1). A similar lack of amplification when utilizing general fungal primers (ITS1 and ITS4) was reported by Pereira *et al* (2014), when the authors attempted to identify fungal symbionts isolated from two Chilean terrestrial orchids, *Chloraea collicensis* and *C. gavilu*. Similarly, amplification was only achieved when *Tulasnella* specific primers (ITS1 and ITS4-Tul) were utilised, with all of the fungal
isolates putatively identified as *Tulasnella* spp. It has been suggested that the general fungal primers (ITS1 and ITS4) are unable to amplify the majority of Tulasnellaceae because of the accelerated evolution that has occurred within their nuclear ribosomal operons (Moncalvo *et al.*, 2000; Binder *et al.*, 2005; Pereira *et al.*, 2014).

Although the orchid genus *Microtis* has been shown to associate with different fungal species (*Tulasnella* spp., *Sebacina* spp. and *Ceratobasidium* spp.) while growing in different environments, the general consensus was that these orchids generally associated with *T. calospora* (Warcup, 1981; Milligan & Williams, 1988; Perkins *et al*., 1995; Bonnardeaux *et al*., 2007; De Long *et al*., 2012). This notion is further supported by the results of this study, which established *M. media* to mainly associate with *Tulasnella* species. Specifically, this study identified isolates obtained from *M. media* plants growing in disturbed bushland as *Tulasnella* spp., which corresponds with the results of the DeLong *et al* (2012) study.

This study has also identified fungal isolates originating from both horticultural beds and from the living collection as *Tulasnella* spp., as opposed to the *Piriformospora indica, Chaetospermum artocarpi, Ceratobasidium* sp or *Sebacina vermifera* reported by De Long *et al* (2012). These differences in fungal species could be attributed to both temporal and sampling site differences, and given that not all of the fungal isolates obtained in this study were successfully identified, the occurrence of other fungal species cannot be ruled out.

In general, our results highlighted the predominant association of *M. media* (growing in disturbed environments) with *Tulasnella*, supporting the notion put forward by Bonnardeaux *et al* (2007), which suggested that *Tulasnella* species were disturbance-tolerant, and occurred in both pristine and disturbed environments. As such, the ability of *M. media* to associate with a widespread and disturbance-tolerant fungal symbiont can be inferred as one of the major adaptations which have allowed for the ruderal expansion of *M. media* into otherwise novel habitats.

4.4.2 Symbiotic germination of *M. media*

Successful germination of *M. media* seed on all of the identified fungal isolates confers functionality of each of the isolates as a true *M. media* symbiont, and also fulfils Koch’s
postulates. Although germination and subsequent protocorm growth of *M. media* was observed with all of the 22 fungal isolates investigated (see Section 4.3.2), isolates varied in their effectiveness at promoting seed germination and protocorm growth. This variation in effectiveness of fungal isolates to promote orchid seed germination and protocorm growth has been reported in many other symbiotic orchid studies (Warcup, 1981; Perkins *et al.*, 1995; Huynh, 2003; Otero *et al.*, 2004; Otero *et al.*, 2005; Otero *et al.*, 2007; Wright, 2007; Wright *et al.*, 2009; Phillips *et al.*, 2011; De Long *et al.*, 2012; Tan *et al.*, 2014). These differences could possibly be due to genetic diversity occurring at the intra-specific level, potentially reflecting functional diversity under varying ecological situations. Specifically, the size differences of *M. media* germinated with different *Tulasnella* isolates (of this study) were very similar to the size differences observed with germinants of *Caladenia formosa* G.W.Carr, when germinated with different isolates of *S. vermifera* (Huynh, 2003; Huynh *et al.*, 2009).

The *in-vitro* observations (of *M. media* germinating with multiple *Tulasnella* sp) of this study potentially suggest that *M. media* is able to associate with multiple *Tulasnella* species in nature, which in turn, reinforces our hypothesis that the ability to associate with widespread and disturbance-tolerant mycorrhizal symbionts has contributed significantly to the ruderal expansion of *M. media*.

Another important observation derived from this investigation would be the apparent lack of correlation between the ability of a fungal symbiont to promote seed germination and its ability to promote seedling growth. Fungal isolates which yielded higher levels of *M. media* germination did not necessarily produce larger seedlings and fungal isolates which yielded larger seedlings did not necessarily have the highest levels of germination. Interestingly, this lack of correlation is not unique to *M. media*, as it has also been reported to occur in another Australian terrestrial orchid, *C. formosa* (Huynh, 2003; Huynh *et al.*, 2009). These results indicate that there are different underlying mechanisms responsible for the promotion of seed germination and seedling growth, which in turn are indicative of genetic variation between isolates. Further work is required to conclusively determine these genetic differences, and their respective effects on seed germination/seedling growth.
Based on the results of this section, which highlighted the following attributes of fungal isolate 2.4.1: a) speed at which *M. media* seeds developed (from seed to stage three protocorms with visible pelotons within ten days of sowing) after inoculation, b) fungal isolate which yielded the best germination of *M. media*, c) development of relatively large protocorms/seedlings (mean lengths of 3.71 ± 0.31 cm, and mean widths of 0.78 ± 0.02 cm), it was decided that all subsequent symbiotic experiments in Chapter 5 and 6 would utilise fungal isolate 2.4.1 (*Tulasnella calospora*) exclusively.

4.4.3 Protocorm and fungal symbiont surface area

Although results have shown *M. media* to germinate rapidly under symbiotic conditions, relatively little is known about how mycorrhizal associations could potentially aid in protocorm nutrition. Specifically, it was intended to determine if mycorrhizal associations expanded the nutritional scavenging abilities of developing protocorms. While fungal hyphal lengths are routinely compared to root lengths in other mycorrhizal investigations (Abbott *et al.*, 1984; Kothari *et al.*, 1991; Li *et al.*, 1991; Rillig *et al.*, 2002; Smith & Read, 2008; Ekblad *et al.*, 2013), the lack of ‘root’ lengths in *M. media* protocorms meant that this option was not available for this study.

The stage four protocorms utilised in this study lacked any roots. In addition, Chapter 3 has already highlighted the lack of any root formation in symbiotically germinated *M. media* seedlings. The lack of root formation was further supported by the investigations carried out in this chapter, as root formation was also not observed in any of the seedlings germinated with the 22 fungal isolates. In the absence of roots, the alternative approach was to compare lengths and surface areas of the mycorrhizal isolates, to the lengths and surface areas of their corresponding protocorms, the protocorm representing the ‘below ground’ structure of the developing orchid and the likely location for nutrient exchange with its fungal symbiont.

Discounting variation between fungal isolates (Table 6), fungal lengths and surface areas averaged about 200 cm and 0.23 cm², respectively, per petri dish. When compared to the minute mean lengths (0.6 cm) and surface areas (0.073 cm²) of the
corresponding protocorms, one can begin to appreciate how associations with mycorrhizal fungi could potentially translate into significant increases in ability to scavenge for nutrients in surrounding substrates. These values (a few magnitudes lower) are nowhere near the hyphal values reported for other mycorrhizal systems (Sanders et al., 1977; Schubert et al., 1987; Abbott et al., 1992; Smith et al., 2004). Nonetheless, these results indicate the potential for the mycorrhizal symbiont to significantly increase the nutrient scavenging capacity of *M. media*. In addition, although these experiments were carried out on a nutrient poor substrate (water agar) in an attempt to mimic nutrient poor soil, they are essentially artificial systems, and future work should include experiments utilizing habitat soil in order to determine if there are any differences in hyphal length/density.

It is well established that both in saprotrophic fungi (Rayner et al., 1994; Rayner, 1996) and in their ectomycorrhizal counterparts (Perez-Moreno & Read, 2000), the vegetative mycelia have the capacity to produce distinctive organisational states associated with the explorative functions which are distinct from assimilative functions. Essentially, exploratory growth over nutrient-poor resources involves the production of fast growing, slender and relatively un-branched mycelial systems that ‘forage’ for nutrient-rich areas. On encountering such areas, hyphal proliferation, sometimes to considerable densities can lead to the production of visible ‘patches’ of mycelial growth. The relatively large hyphal densities reported in the literature for arbuscular mycorrhizal (Schubert et al., 1987; Abbott et al., 1992; Smith et al., 2004) and ectomycorrhizal (Rousseau et al., 1994; Perez-Moreno & Read, 2000) systems represent such changes of organisational state and have generally been measured using mycelium growing on relatively enriched substrates such as soil or plant litter. Seen in this light, the lower densities of hyphal development observed in the orchid mycorrhizal fungus growing on water agar, in the present study, may not be surprising since these systems would be in the exploratory mode of hyphal growth. It remains to be determined whether orchid mycorrhizal fungi show the same flexibility of organisational state as seen in other mycorrhizal types.

Unexpectedly, this investigation into hyphal lengths/surface area has potentially shown a trend between protocorm size and fungal growth, the better growing fungal isolates (in terms of fungal length) producing larger protocorms. As previous sections
of this chapter have already shown *Tulasnella* isolates vary in their abilities to promote germination and growth. Subsequent corresponding differences in protocorm fitness are therefore, not surprising. Interestingly, these differences in protocorm size occurred within a week, as protocorms of similar sizes from all three fungal isolates were chosen (at the start of the investigation) before being transferred onto water agar. Given that this investigation was conducted in the dark, and that non-photosynthetic stage four protocorms were utilized, one can confidently rule out any effects of photosynthesis on protocorm growth. Any differences in protocorm size can therefore be attributed as being solely due to the effects of mycorrhizal fungi. Further detailed studies would be required to positively establish this trend.
4.5 Conclusions

The mycorrhizal symbionts associating with ruderal poplations of *M. media* have been characterised by the investigations carried out in this chapter. Specifically, the following points have been established:

- Majority of fungal symbionts have been determined as *Tusnella* species
  - All of which are able to promote *M. media* seed germination and subsequent protocorm growth to varying degrees
    - Indicative of the ability of *M. media* to associate with multiple *Tulasnella* species
  - The isolation of *Tulasnella* species from *M. media* plants growing within disturbed environments
    - Indicative of those *Tulasnella* isolates being both widely distributed and disturbance-tolerant
  - The ability of *M. media* to associate with multiple very widely distributed and disturbance-tolerant *Tulasnella* genotypes can therefore be inferred as a significant adaptation contributing to its success as a ruderal species.

- Preliminary baseline values of hyphal length and surface area of mycorhizal fungi
  - Suggest that associations with mycorrhizal fungi should significantly increase the abilities of developing protocorms to scavenge for nutrients from surrounding substrates.

Thus, the results reported in this chapter support HYPOTHESIS 3: That particular taxonomic and eco-physiological attributes of the mycorrhizal fungal associations of *M. media* will promote successful seedling establishment under in-vitro, and *in-situ* conditions. Building on the results of this chapter, the next chapter aims to determine how associating with mycorrhizal fungi aids in the nutrition (phosphorus) of the developing protocorm.
4.6 References


Chapter 5: Phosphorus nutrition of *M. media*
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5. Abstract

As will be discussed in the following paragraphs, *M. media* is naturally distributed across habitats that are inherently nutrient impoverished, especially in the essential nutrient phosphorus. Investigations into the phosphorus nutrition of *M. media* have elucidated a few key adaptations which would be expected to contribute to the ruderal habit of the species. Elevated concentrations of phosphorus were detected within *M. media* seeds, and can be inferred as an adaptation of the species to maximise offspring survivability. Phosphorus contents of *M. media* were observed to increase with developmental stage, and this increase can be mainly attributed to the provision of phosphorus by mycorrhizal fungi. From the microcosm experiments, most of the mycorrhizal symbionts were identified as *Tulasnella* species, supporting the hypothesis that *M. media* predominantly associates with members of the genus *Tulasnella*. In addition, the abilities of these mycorrhizal symbionts to access (take-up) inorganic and organic forms of phosphorus (non-radioactive and radioactive forms), and subsequently transfer them into developing *M. media* (protocorms and adults), have also been established. The ability of *M. media* to access and utilise both inorganic and organic forms of phosphorus, by way of their mycorrhizal symbionts can therefore be inferred as one of the major adaptations which have allowed for the successful expansion of the species.

5.1 Introduction

*Microtis media* occurs widely as a component of natural, semi-natural and ruderal plant communities across Western Australia (see Figure 2.1b in Chapter 2). Amongst its natural habitats are sand plains that extend across the south-west of the state. The sandplain communities, of which the orchid is a component, are known locally as the ‘Kwongkan’ or ‘Kwongan’, and are internationally renowned for their extreme floristic diversity. They comprise part of what is recognised as the South-west Australian Floristic Region (SWAFR) (Hopper & Gioia, 2004; Hopper, 2009). Defining features of the soils of the region are their extreme nutrient deficiency (McArthur, 1991; Lambers et al., 2008; Lambers, 2014), and the fact that of all the known elements limiting plant
growth, it is phosphorus, rather than nitrogen, which is limiting plant growth in this region (Lambers, 2014).

The causes of the extreme nutrient limitation have been extensively discussed (see Mucina et al in Lambers 2014) and it is widely agreed that the great age of the parent materials of the soils is a major contributing factor. The Kwongan soils are derived from substrates which have been subject to extreme weathering since Permian times (c 260 million years) (Hopper & Gioia, 2004; Hopper, 2009) and in the absence of rejuvenating events such as glaciaion or volcanism, there has been a progressive diminution in the original stocks of phosphorus, as well as of numerous other essential micronutrients.

Since the floristic diversity of these old, climatically buffered infertile landscapes (OCBIL sensu Hopper 2009) makes them of international conservation importance, there is much interest in the eco-physiological adaptations that have enabled plants to cope with the extreme nutrient impoverishment, with special emphasis being placed on their phosphorus nutrition. In this context, much progress has been made towards elucidating the specialised mechanisms involved in the phosphorus acquisition by the often dominant non-mycorrhizal components of the SWAFR, in particular the Proteaceae (Lamont, 2003; Lambers et al., 2008).

Members of this family, together with those of the unrelated families Cyperaceae (Juss) and Restionaceae (R.Br) produce ‘cluster roots’ sensu Lamont (2003), and/or dauciform or ‘carrot’ shaped roots (sensu Lamont 1974). Both forms (of roots) are essentially repeatedly branched fine roots that are densely covered with root hairs, and aid in the phosphorus nutrition of the plants by producing vast amounts of root exudates such as carboxylates. Carboxylates facilitate the solubilisation of phosphorus which are sorbed onto the surface of soil particles, and increases the amount of free phosphorus available for plant uptake (Lamont, 1974; Shane et al., 2004; Shane et al., 2005; Lambers et al., 2006; Playsted et al., 2006).

Interestingly, the solubilisation of sorbed phosphorus from soil particles by the exudation of carboxylates is not limited to plants with cluster or dauciform roots, as carboxylate production has also been reported in a few members of the Fabaceae (Lindl) occurring within the SWAFR (Ryan et al., 2012). Mycorrhizal systems of the
ericoid, ectomycorrhizal and arbuscular kinds, which are all known to have the potential to aid in the capture of phosphorus (Smith & Read, 2008), are also widely found in plants of the SWAFR, but their functional attributes in these OCBIL landscapes remain largely unknown. At the broader level, ericoid (Van Leerdam et al., 2001), ectomycorrhizal (Finlay & Read, 1986) and arbuscular (Jakobsen et al., 2001) mycorrhizal fungi have been shown to produce exudates which facilitate the utilisation (and subsequent transfer to host plant) of both organic and inorganic forms of phosphorus present within the soil (van Schöll et al., 2008; Martino & Perotto, 2010).

Orchids are recognised to contribute a significant component of the biodiversity of the SWAFR, with upwards of 400 species having been described in these ecosystems (Brown et al., 2008). Despite their presence as a significant component of this vitally important ecosystem, there has been no study of the phosphorus nutrition of this group of plants. Although orchid seed nutrient has been investigated in a few early studies (Beyrle et al., 1991; Richardson et al., 1992; Beyrle & Smith, 1993; Beyrle et al., 1995), the phosphorus nutrition of orchids, and in particular, the role of orchid mycorrhiza in the phosphorus nutrition of their host orchids has been neglected. There have been three studies involving the phosphorus nutrition of orchids of the Northern hemisphere (Smith, 1966; Alexander et al., 1984; Cameron et al., 2007), but none to date of any orchid species of the Southern hemisphere. The Northern hemisphere studies have confirmed the potential of the mycorrhizal associates of two different orchid species to capture phosphorus from the growth medium and transfer it to the plant.

Nitrogen, which exists as both organic (amino acids, peptides and proteins) and inorganic (ammonium and nitrate) forms within soil, is generally accepted as the main nutrient limiting plant growth in most younger ecosystems (Aber, 1992; Chalot & Brun, 1998; Knicker & Skjemstad, 2000; Smith & Read, 2008; Hopper, 2009). Availability of nitrogen (within soil), has also been shown to influence phosphorus uptake in plants (Arnon, 1939; Olson & Dreier, 1956; Grunes, 1959; Leonce & Miller, 1966; Riley & Barber, 1969; Riley & Barber, 1971; Marschner & Marschner, 2012).

In view of the fact that throughout the juvenile stages of their lives, orchids have few, if any roots which can be utilised in nutrient capture, it can be hypothesized that in
nature, and particularly in environments such as the SWAFR (characterized by their extreme phosphorus deficiencies), capture of phosphorus by mycorrhizal fungi would be essential for survival. This hypothesis is examined here using *Microtis media* as the test orchid species.

### 5.1.1 Aims

Given that relatively little is known about the phosphorus nutrition of *M. media*, this chapter aims to improve our understanding of *M. media* by investigating the following:

- **Baseline phosphorus concentrations of *M. media* in different life stages**
  - Determination of baseline (P) contents and tissue P concentrations of *M. media* seeds, protocorms and whole plant organs (Experiment 5.1)

- **Effects of organic and inorganic forms of P, each supplied at a range of concentrations (0, 2, 4, 6, 8 and 10 ppm) upon P accumulation within *M. media* seedlings (Experiment 5.2.1) and upon extension growth of the fungal symbiont *T. calospora* – isolate 2.4.1 on nutrient agar (Experiment 5.2.2).**

- **Effects of supplementation of growth media with different forms (organic and inorganic) and concentrations (0, 20, 40, 60, 80, 100 ppm) of nitrogen upon P accumulation in *M. media* seedlings (Experiment 5.3).**

- **Analysis of the uptake and transfer of radioactively labelled ($^{33}$P) supplied either as inorganic $^{33}$P orthophosphate or as organic $^{33}$P inositol polyphosphate ability, by the mycorrhizal fungus to protocorms (Experiment 5.4) and adult plants (Experiment 5.5) of *M. media*.
5.2 Materials and methods

Experiment 5.1 – Determination of baseline P contents and tissue P concentrations of *M. media* seeds, protocorms and whole plant organs.

5.1.1 – Digestion of tissue samples and colorimetric determination of P contents of *M. media* seed, protocorms and plants.

The digestion protocol involved the addition of a pre-weighted amount of plant material (seeds (5 lots of 1000 seeds each), stage three (300 protocorms) and five protocorms (100 protocorms), and ~ 50mg for adult plant material) into an acid washed glass digest tube, followed by the addition of approximately 50 mg of catalyst (lithium sulphate: copper sulphate in a 10:1 ratio) and 4 mL of the sulphuric/salicylic acid mix (33 g of salicylic acid dissolved in one litre of concentrated sulphuric acid). The digest tubes were then heated to 370 °C in a heating block, for approximately 6 hours, or until the solution went clear. Following that, the tubes were removed from the heating block, allowed to cool, before being diluted to a final volume of 50mL.

Phosphorus concentrations were determined by a colorimetric method (Watanabe & Olsen, 1965). Solutions for analysis (3.8 ml) were made up in disposable plastic cuvettes, comprising of 0.5 ml digested sample, 0.5ml development solution (100mL of ammonium molydbate [4 g dissolved in 100 ml of distilled water], 25ml of antimony potassium tartrate [1.454g dissolved in 500ml of distilled water], added to 250mL of 2.5M sulphuric acid, and diluted to a final volume of 500ml with distilled water), 0.2mL of 0.5M ascorbic acid (0.88g in 10ml distilled water, prepared fresh as solution does not keep beyond 24hours), and 2.6ml of distilled water. Samples were allowed to develop for 30 minutes before measurements of absorbance at a wavelength of 882 nm were carried out with a spectrophotometer. A series of external phosphorus (sodium hydrogen orthophosphate) standards (0.5 to 6 mg ml⁻¹) was also prepared and used to quantify the phosphorus concentrations within the digested samples (Figure 5.1).
5.1.1.i Seeds

*Microtis media* seeds were collected from mature capsules of *M. media* growing in bushland (Kings Park, Perth, Western Australia), dried in bulk and stored in a desiccator until use. Five sub-samples of the bulked seed, each consisting of 1000 seeds (± 20) were selected for analysis of their total P concentration using the method described above.

5.1.1.ii Protocorms

Seeds were surface sterilised as described in Chapter 2, and plated onto 0.25 % oatmeal agar in the presence of fungal isolate 2.4.1 - *T. calospora*. The plates were incubated at 15 °C under complete darkness until the protocorms had developed and reached stages three and five (see Table 3.2 in Chapter 3). They were then harvested, oven dried at 50 °C for seven days and digested as bulked samples of 20 protocorms prior to analysis of their P contents.
5.1.1.iii Adult plant tissues

Five adult plants were collected from native bushland soils within Kings Park, Perth, Australia, and separated into leaf, root and tuber. These were then oven dried as above before tissue P determination.

**Experiment 5.2.1** – Determining the differences and effects of exposure to organic and inorganic forms of P, on the P accumulation of *M. media* protocorms

Two identical sets of experiments were carried out concurrently, with one set investigating the effects of different phosphorus sources on *M. media* protocorm accumulation (Experiment 5.2.1), and the other investigating the effects of different phosphorus sources on extension growth of the mycorrhizal symbiont, *T. calospora* (Experiment 5.2.2). Stage three symbiotically germinated (with *T. calospora*, fungal isolate 2.4.1) *M. media* protocorms (n = 50 per treatment, 10 per harvest) were utilised in this experiment.

The basal P-free medium used for this experiment was a modified BM medium (Van Waes, 1984; Rasmussen, 1995) containing: 0.606 g \( \text{L}^{-1} \) ammonium nitrate, 0.14 g \( \text{L}^{-1} \) magnesium sulphate, 50 mg \( \text{L}^{-1} \) calcium chloride, 25 mg \( \text{L}^{-1} \) sodium chloride, 164 mg \( \text{L}^{-1} \) potassium chloride, 3 mg \( \text{L}^{-1} \) zinc sulphate, 12.5 mg \( \text{L}^{-1} \) Ferric EDTA, 0.13 mg \( \text{L}^{-1} \) Thiamine, 1 mg \( \text{L}^{-1} \) copper sulphate, 20 mg \( \text{L}^{-1} \) iron sulphate, 1 mg \( \text{L}^{-1} \) sodium molybdite di-hydrate, 10 mg \( \text{L}^{-1} \) boric acid, 10 mg \( \text{L}^{-1} \) manganese sulphate; 10 mg \( \text{L}^{-1} \) zinc sulphate, 5 g \( \text{L}^{-1} \) glucose, 6 g \( \text{L}^{-1} \) agar, pH 6.5 before autoclaving, was utilised as a basal growth medium. The basal medium was supplemented with either organic P in the form of phytic acid at a range of concentrations from 0, 2, 4, 6, 8 and 10 ppm, or inorganic P in the form of mono-potassium phosphate at a range of concentrations from 0, 2, 4, 6, 8 and 10 ppm.

Protocorms were incubated under light conditions (see Chapter 2) at 15 °C, and harvests were carried out every 20 days, with the experiment running for a total of 80 days. All samples were oven dried at 50 °C for seven days, weighed and analysed for their phosphorus contents with the colorimetric method described previously. Due to their minute final dry weights, individual samples had to be pooled into their respective treatments before analysis (in order to meet the minimum weight requirements) and as such, no statistical analysis could be carried out.
**Experiment 5.2.2** Determination of the effects of inorganic and organic sources of P upon the extension growth of *T. calospora* on agar

Individual stage three protocorms (*n* = 10) were transferred onto petri-dishes containing BM medium which had been supplemented with the organic and inorganic concentrations described above (Experiment 5.2.1). Positions of the hyphal tips of extending hyphal fans were marked at weekly intervals for a period of three weeks, and the extension growth obtained over the intervening periods determined.

**Experiment 5.3** Effects of supplementation of the growth medium with different forms and concentrations of nitrogen (N) upon tissue P concentrations of *M. media* protocorms

Stage three *M. media* protocorms (*n* = 50 per treatment, 10 per harvest) generated symbiotically with fungal isolate 2.4.1 (*T. calospora*) were utilised in this experiment. The basal low nitrogen medium used for this experiment was a modified BM medium containing: 0.3 g \( L^{-1} \) mono-potassium phosphate, 0.25 g \( L^{-1} \) ammonium phosphate, 0.14 g \( L^{-1} \) magnesium sulphate, 50 mg \( L^{-1} \) calcium chloride, 25 mg \( L^{-1} \) sodium chloride, 3 mg \( L^{-1} \) zinc sulphate, 12.5 mg \( L^{-1} \) Ferric EDTA, 0.13 mg \( L^{-1} \) Thiamine, 1 mg \( L^{-1} \) copper sulphate, 20 mg \( L^{-1} \) iron sulphate, 1 mg \( L^{-1} \) sodium molybdate di-hydrate, 10 mg \( L^{-1} \) boric acid, 10 mg \( L^{-1} \) manganese sulphate, 10 mg \( L^{-1} \) zinc sulphate, 5 g \( L^{-1} \) glucose, 6 g \( L^{-1} \) agar, pH 6.5 before autoclaving was utilised as a basal growth medium. The basal medium was supplemented with either organic N in the form of L-glutamine at a range of concentrations from 0, 20, 40, 60, 80 and 100 ppm, or inorganic N in the forms of calcium nitrate, potassium nitrate and ammonium sulphate, at a range of concentrations from 0, 20, 40, 60, 80 and 100 ppm.

Protocorms were incubated under light conditions (see Chapter 2) at 15 °C, and harvests were carried out every 20 days, with the experiment running for a total of 80 days. All samples were oven dried at 50 °C for seven days, weighed and analysed for their phosphorus contents with the colorimetric method described previously. Due to their minute final dry weights, individual samples had to be pooled into their respective treatments before analysis (in order to meet the minimum weight requirements) and as such, no statistical analysis could be carried out.
Experiment 5.4 Petri-dish experiments involving *M. media* protocorms and radioactively labelled inorganic and organic forms of phosphorus

5.4.1 Radioisotope sources

Pots of $^{33}$P labelled orthophosphoric acid (37 MBq of activity, inorganic phosphorus) was purchased from Perkin Elmer (Massachusetts, USA).

As $^{33}$P labelled inositol polyphosphate (organic phosphorus) is not commercially available, it was synthesized by the School of Chemistry, University of Sheffield, UK. Its synthesis involved the charging of a test tube equipped with a stirring bar and condenser with 90 mg (0.5 mmol) of myo-inositol, 156 mg (1.6 mmol) of $^{33}$P labelled \( \text{H}_3\text{PO}_4 \), 0.4 ml (1.6 mmol) \( \text{Bu}_3\text{N} \) and 1 ml of DMF (Dimethylformamide). The mixture was stirred overnight in a preheated heating block at reflux (200 °C). The reaction mixture was cooled to room temperature, and the solvents were removed under reduced pressure using a rotary evaporator (80 °C). The crude mixture was diluted with a minimum amount of MeOH (methanol) and directly purified by an ion exchange resin (Cl – form, DEAE sepharose), before being eluted with 400 ml of MeOH then 300 ml of MeOH/1M HCL (1/1). The acidic eluents were collected and concentrated using a rotary evaporator (80 °C) to afford the desired $^{33}$P labelled inositol polyphosphate.

Structural makeup of labelled inositol polyphosphate was confirmed with high resolution mass spectrometry (HRMS): (ES) \( m/z \ [M + H]^+ \) calculated for \( \text{C}_6\text{H}_{14}\text{O}_9\text{P}_1 \) 261.0375, found 261.0376; calculated for \( \text{C}_6\text{H}_{15}\text{O}_{12}\text{P}_2 \) 341.0039, found 341.0049; calculated for \( \text{C}_6\text{H}_{16}\text{O}_{15}\text{P}_3 \) 420.9702, found 420.9683; calculated for \( \text{C}_6\text{H}_{17}\text{O}_{18}\text{P}_4 \) 500.9365, found 500.9384; Melting point = 30 – 31 °C. Purity and the absence of free phosphate ions was reaffirmed by nuclear magnetic resonance (NMR); 1H NMR (400 MHz, CDC\( \text{l}_3 \)): \( \delta \) 4.55 – 3.22 (18H, m, CH and P(O)(OH)\(_2\)); 13C NMR (100.6 MHz, D\(_2\)O): \( \delta \) 79.3, 73.7, 72.3, 72.1, 71.2; 31P NMR (400 MHz, CDC\( \text{l}_3 \)): \( \delta \) -0.06 to -0.67 (m).

5.4.2 Experimental setup

Protocorms germinated with *T. calospora* (fungal isolate 2.4.1) were transferred individually into split petri-dishes (Figure 5.2.a) containing water agar (6 g\(^{-1}\)) and incubated for ten days to allow the fungal symbiont to extend over the agar on both sides of the dish. Stage five protocorms (\( n = 15 \)) were utilised for the inorganic $^{33}$P (\( ^{33} \)P
labelled orthophosphoric acid) experiments, while stage three (n = 15) and four (n= 15) protocorms were utilised for the organic \(^{33}\text{P}\) \((^{33}\text{P} \text{ labelled inositol polyphosphate})\) experiments.

A 10 mm disc of agar was then removed from the fungus-only compartment of the dish, and was replaced with a slightly smaller (8 mm) disc of the same agar (containing either 115 pico-grams of inorganic \(^{33}\text{P}\), or 111 picograms of organic \(^{33}\text{P}\)). The use of a disc of smaller diameter as the isotope source enabled the formation of an agar-free diffusion barrier around the introduced source disc. Control dishes (n = 15) were also set up, in which a 5 mm strip of agar was removed across the plates, so breaking all the mycelial connections between the P source and the protocorms.

Following introduction of the isotope, dishes were incubated for 12 days under light conditions at 15 °C. After this incubation period, the agar disc containing the radioisotope was removed (to avoid quenching effects during imaging), before the agar sheets supporting the fungal mycelia were lifted intact from the petri-dishes, and laid onto a Perspex backing sheet. This was then inserted into the scanning chamber of a Packard Instant Imager (Isotech, Chesterfield, UK), to obtain real-time views of the distribution of radioactivity in the systems.

5.4.3 Determination of tissue \(^{33}\text{P}\) content by liquid scintillation counting

Protocorms were oven dried (80 °C for 3 days) and weighed. Individual dry protocorms were then placed into acid-washed digest tubes, to which 1 ml concentrated sulphuric acid was added. Tubes were then allowed to sit at room temperature for 3 hours, before placing them into a pre-heated heating block (350 °C, Grant instruments, Cambridgeshire, UK) for ten minutes. After heating, the orchid tissue had dissolved into black carbonised slurry. Tubes were then removed from the heating block and allowed to cool for five minutes before 0.2 ml of 100 volumes hydrogen peroxide was added. Tubes were returned to the heating block for a further five minutes. If the carbonised slurry had not cleared to a colourless solution after this step, the process (addition of more hydrogen peroxide and heating) was repeated until a colourless solution was achieved. Following that, the samples were allowed to stand at room temperature for 24 hours to allow for the decomposition of any residual hydrogen peroxide before being made up to a final volume of 10 ml with distilled water. A 1 ml
 aliquot was mixed with 10 ml of emulsifying scintillant (Ecosafe, Fisher Biosciences, UK) before liquid scintillation counting for $^{33}$P (Packard Tri-Carb 3100TR; Isotech, Chesterfield, UK). Calculations into the $^{33}$P content of protocorms were carried out as per Cameron et al (2006) after correcting for radioactive decay.

**Experiment 5.5** Microcosm experiments involving *M. media* adults and radioactively labelled inorganic and organic forms of phosphorus

The mycorrhizal fungi associating with these adult *M. media* plants were determined to be mostly *Tulasnella* species. Fungal sampling and molecular identification procedures, together with all of the fungal identities are described in Appendix 1.

5.5.1 Inorganic phosphorus

Dormant *M. media* tubers (n = 40) were collected (summer of 2013) from bushland within Kings Park, together with the surrounding habitat soil. Dormant tubers were potted up with habitat soil, and revived under glasshouse conditions with the onset of autumn in 2014. Plants were allowed to grow for six weeks (still in a stage of active shoot growth) before they were removed from their pots. Roots were cleaned of soil particles under a flowing stream of water before being introduced into an agar microcosm (Figure 5.2 b).

Cleaned *M. media* plant (roots) were laid on the agar surface of the microcosm, and secured using approximately 5 ml of cooled molten water agar (applied to the roots). Microcosms were incubated under light conditions at 15 °C for two weeks, in order to allow for fungal growth to occur across the entire microcosm.

The microcosms used in this experiment consisted of 25 cm square transparent acrylic boxes of 2 cm depth. A groove was cut in the upper vertical wall of each microcosm to allow for the protrusion of the shoot, together with a 1 cm window, which was also cut distal (~ 8 to 10 cm) to the groove to permit the insertion of mesh lined tubes containing soil. The tubes were constructed following the recommendations of Johnson et al (2001), and consisted of 10 cm lengths of PVC pipe (internal diameter 18 mm). Two ‘windows’ were cut along each side of each pipe, these windows being covered with nylon mesh of a pore size (35 microns) sufficient to enable passage of hyphae, while excluding roots. The mesh was sealed to the tubes using PVC cement
The tubes were filled with bushland soil collected from the natural habitat of *M. media*, and then sealed into the microcosms. The boxes were then filled to approximately 1 cm depth with 150 ml of molten water agar (6 g agar⁻¹). Orchid plants were placed upon the surface of the agar with their roots at a minimum distance of 1 cm from the soil tube. See Figure 5.2c for experimental set up.

Of the microcosms set up, 18 were subjected to hyphal breakage treatment (by twisting of soil cores 90° along their axis every second day, after isotope application), and 18 were left intact (soil cores left un-twisted). Each soil core was injected with 0.5 MBq of ³²P labelled orthophosphoric acid with a sterile 1 ml syringe attached to a sterile needle. The isotope was only released from the syringe as the needle was being withdrawn from the soil core, thus ensuring that the isotope was distributed in the middle and along the axis of the soil core.

Harvests (n = 3 for each treatment, total of 6 per harvest point) were carried out every second day, for a total of six harvests (12 days). The agar sheets supporting the fungal mycelia were removed intact from the acrylic boxes after the incubation periods, and laid onto a Perspex backing sheet, and inserted into the scanning chamber of a Packard Instant Imager (Isotech, Chesterfield, UK), to obtain real-time views of the distribution of radioactivity in the systems. Plant tissues were harvested (n = 3 for each treatment, total of 6 per harvest point) every second day, for a total of six harvests (12 days), before being prepared for liquid scintillation counting using the protocols outlined above (Section 5.4.3).

5.5.2 Organic phosphorus

In order to determine whether adult *M. media* plants could access organic P, microcosms of the type described in 5.5.1 were employed. However, in this experiment, to each microcosm, approximately 30 g of moist pasteurised soil (from *M. media* habitat) was added directly a channel cut in the water agar of microcosms (see Figure 5.2.c). At the same time as soil was added, adult *M. media* plants (n = 5) were introduced to the microcosm and these systems were incubated for two weeks to allow growth of the fungal symbiont from the orchid, across agar and into the soil filled channel. After this initial incubation period, 0.5 MBq of ³²P inositol polyphosphate was
added directly to the centre of the soil channel using a sterile syringe. Because of limited availability of the isotope, only one microcosm could be utilized as the control. In this, a 5 mm wide agar channel was cut between the orchid roots and the soil channel prior to isotope application, in order to sever all hyphal connections with the soil channel.

Following isotope application, these microcosms were incubated for a further period of 12 days before plants were harvested and their $^{33}$P contents were determined as described in section 5.4.3.

**Figure 5.2** Overview of $^{33}$P experiments, highlighting the positions of the orchid (i), and the location where the isotope was applied (ii and arrows). **a.** Experimental setup investigating the ability of the mycorrhizal symbiont to access either inorganic or organic forms of phosphorus ($^{33}$P labelled orthophosphoric acid or inositol polyphosphate). *Microtis media* protocorms (i) were introduced into one end of a split Petri-dish, and an agar disc containing 0.5 MBq of $^{33}$P was introduced into the other end. **b.** Experimental setup investigating the effects of hyphal breakage on phosphorus uptake in *M. media* (i). Each system was supplied with 0.5 MBq of $^{33}$P labelled orthophosphoric acid, introduced by injection (white arrow) into the soil core (ii, containing habitat soil). **c.** Experimental setup investigating the ability of the mycorrhizal symbiont to access organic phosphorus applied directly to habitat soil. 0.5 MBq of $^{33}$P labelled inositol hexaphosphate was applied (to the habitat soil filled agar channel highlighted by the arrow) to each microcosm.

5.2 Data analysis
Data was checked for conditions of normality and homogeneity of variance using the Kolmogorov-Smirnov Test and Levene’s Test. For data that conformed to the conditions, one way Analysis of Variance (ANOVA) was used to analyse for the effect of the factor on the variable. When an effect was detected, pairwise comparisons were performed with Dunnett’s C. For data that did not conform to the conditions even after transformations, non-parametric methods (Kruskal Willis and Mann-Whitney U tests) were used.

Results from experiment 5.5 yielded no statistical differences when pairwise comparisons were carried out. As such, in order to understand the mechanism of phosphorus uptake, a three - parameter log-logistical function was used to fit phosphorus accumulation data related to harvest times using the ‘drc’ package in R (Ritz & Streibig, 2005),

\[ F(x, b, d, e) = \frac{d}{1 + \exp[b(\log(x) - \log(e))]} \]

whereby \((d)\) is the parameter for the upper limit or maximum phosphorus accumulation on the curve, \((b)\) proportional to the \textit{slope} of the curve of \(F\) and harvest point \((x)\), and the parameter \((e)\) equal at the median point (= slope inception). A three parameter log-logistical curve was chosen as it was most suitable for determining the responses over other non-linear functions. The curve selection was based on the lowest Akaike Information Criterion (AIC = -278), as described in Ritz and Streibig (2005). Comparison of calculated parameters was performed through pairwise comparisons of means, as described in Ritz and Streibig (2005). All analyses were conducted in R (R Development Core Team 2013).
5.3 Results

5.3.1 Experiment 5.1 - Baseline P contents and concentrations in *M. media*

Seed tissue contained the highest concentrations of P (Table 5.1). While protocorm tissues had lower concentrations of P, there was evidence of doubling of their P concentrations between stages three and five of protocorm development. In field collected adult plant tissues, leaves and roots showed similar P concentration, in both cases the values being higher than those observed in the laboratory-grown protocorms (Table 5.1). In both concentration and content terms, the P values of the tubers were approximately half of those observed in the leaves and roots. Analysis of the P content data (Table 5.1) reveals a progressive increase in P acquisition through protocorm stage three and five relative to the values calculated for the seeds. Between stages three and five, there is nearly a hundred times increase in P content of the protocorms.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Phosphorus concentration ± standard error (µg g⁻¹ DW)</th>
<th>Phosphorus content ± standard error (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seed</td>
<td>2970.53 ± 269.98</td>
<td>0.0029 ± 0.0003</td>
</tr>
<tr>
<td>Stage 3 protocorm</td>
<td>669.87 ± 250.56</td>
<td>0.0195 ± 0.007</td>
</tr>
<tr>
<td>Stage 5 protocorm</td>
<td>1359.68 ± 219.11</td>
<td>1.7701 ± 0.3075</td>
</tr>
<tr>
<td>Adult (Total plant)</td>
<td>1725.53 ± 190.24</td>
<td>228.257 ± 24.0409</td>
</tr>
<tr>
<td>Adult leaf</td>
<td>2096.43 ± 156.98</td>
<td>110.4406 ± 7.5587</td>
</tr>
<tr>
<td>Adult root</td>
<td>2188.58 ± 160.34</td>
<td>60.7280 ± 13.0865</td>
</tr>
<tr>
<td>Adult tuber</td>
<td>1428.31 ± 169.30</td>
<td>57.0881 ± 9.7390</td>
</tr>
</tbody>
</table>
5.3.2 Experiment 5.2.1 - Determining the differences and effects of exposure to organic and inorganic forms of P, on the P accumulation of *M. media* protocorms

The weights of individual protocorms at all harvests were so low that replicates had to be pooled within their respective treatments to achieve the minimum weights required for analysis. As a result, the statistical significance of the data could only be determined at the 5% level. The phosphorus concentrations of *M. media* seedlings harvested over the course of this study indicate the preferential utilization of the inorganic phosphorus form, over the organic form (Figure 5.3).

There were no clear trends in response to the supply of organic P (Figure 5.3 a). Step-wise increase in supply of organic P from 0 to 10 ppm led to no increase in the P concentration. Indeed, at 10 ppm, P yields were lower than at zero added P at all harvests. See the discussion of this chapter for further consideration of these results.

In contrast, in the case of inorganic P, increase in supply of the element did lead to a progressive increase in the P contents of the developing protocorms (Figure 5.4 b). The greatest increase in P content occurred between 0 and 2 ppm of added P, indicating that at zero P, availability of P was a factor limiting protocorm development. See discussion for further evaluation of these results.
Figure 5.3 Phosphorus concentrations (µg g⁻¹ dry weight) of protocorms grown on different concentrations and forms of phosphorus, harvested every 20 days. 

a. Seedlings grown on different concentrations (0, 2, 4, 6, 8, 10 PPM) of organic phosphorus (phytic acid).

b. Seedlings grown of different concentrations (0, 2, 4, 6, 8, 10 PPM) of inorganic phosphorus (KH₂PO₄). Error bars denote 5% standard error.
Figure 5.4 Mean phosphorus contents of *M. media* protocorms (µg) grown on different concentrations and forms of phosphorus, harvested every 20 days. **a.** Seedlings grown on different concentrations (0, 2, 4, 6, 8, 10 PPM) of organic phosphorus (phytic acid). **b.** Seedlings grown of different concentrations (0, 2, 4, 6, 8, 10 PPM) of inorganic phosphorus (KH₂PO₄). Error bars denote 5% standard error.

### 5.3.3 Experiment 5.2.2 - Determination of the effects of inorganic and organic sources of P upon the extension growth of *T. calospora* on agar

Significant differences in daily growth rates were observed between the inorganic and organic phosphorus treatments, across all concentrations (except the controls, Figure 5.5). In contrast to what was observed from the plant growth experiments, it was the organic phosphorus treatments which yielded greater fungal growth. No ‘optimal’ concentration allowing for better fungal growth was observed, as fungal growth rates were somewhat similar across the different organic phosphorus concentrations. This was also observed in the inorganic phosphorus treatments, with different concentrations yielding somewhat similar growth rates.
5.3.4 Experiment 5.3 - Effects of addition of organic and inorganic forms of nitrogen supplied over a range of concentrations on the abilities of *M. media* protocorms to acquire P.

As in the case of Experiment 5.2.1, it was necessary to pool the protocorm weights in order to determine their P concentrations and total P contents. In terms of both P content (Figure 5.6) and concentration (Figure 5.7), only the addition of increasing quantities of the ammonium ion led to greater P accumulation (Figures 5.6 c and Figure 5.7 c). Indeed, with increasing concentrations of the two nitrate sources (Figure 5.6 a,b and Figure 5.7 a,b), and of the organic source, glutamine (Figure 5.6 d and Figure 5.7 d), there was a trend either for no effect upon P gain, or for an inhibitory impact. The latter was particular marked in the case of substrate supplemented with glutamine. See discussion for further evaluation of these results.
Figure 5.6 Phosphorus concentrations (µg g\(^{-1}\) dry weight) of *M. media* protocorms grown on different concentrations and forms of nitrogen (a: Ca(NO\(_3\))\(_2\), b: KNO\(_3\), c: (NH\(_4\))SO\(_4\), d: glutamine) of nitrogen, harvested every 20 days, for a total of 80 days. Error bars denote 5% standard error.
Figure 5.7 Phosphorus contents (µg) of *M. media* protocorms grown on different concentrations and forms of nitrogen (*a*: Ca(NO₃)₂, *b*: KNO₃, *c*: (NH₄)SO₄, *d*: glutamine) of nitrogen, harvested every 20 days, for a total of 80 days. Error bars denote 5% standard error.
5.3.5 Experiment 5.4 - Transfer of inorganic and organic forms of $^{33}$P to protocorms of *M. media* in petri-dishes

The mycelia of *T. calospora* were readily able to cross the 2.00 mm diffusion barriers in systems supplied with either inorganic (Figure 5.8 a,b) or organic (Figure 5.9 a,b) forms of P, and to translocate the labelled element across this and the plastic central barrier to protocorms of *M. media*, from which they were growing. There is evidence (Figure 5.8 b and 5.9 b) of different patterns of P transfer in the two types of P source, the inorganic (mineral) P being more mobile within the mycelial network than the organic. This may account for the apparently greater quantities of P accumulation in the protocorms supplied with inorganic form compared with the organic form of the element (Table 5.2). However, direct statistical comparisons cannot be made between the two sources because the protocorms involved were of different developmental stages. No $^{33}$P was detected within protocorms of the control treatments, indicating that it was the mycorrhizal symbiont that was responsible for the provision of $^{33}$P.

Table 5.2 The content of $^{33}$P (supplied either as $^{33}$P orthophosphoric acid or $^{33}$P inositol polyphosphate) in *M. media* protocorms of different stages, grown in petri-dish systems. Significant differences in $^{33}$P content were observed in *M. media* protocorms (of different stages) grown with $^{33}$P inositol polyphosphate ($K$=14.5, $P = 0.001$), and are highlighted by different letters.

<table>
<thead>
<tr>
<th>M. media life stage</th>
<th>Content of $^{33}$P</th>
<th>Content of $^{33}$P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>± Standard error (pg)</td>
<td>± Standard error (pg)</td>
</tr>
<tr>
<td>Protocorm: Stage three</td>
<td>0.00010 ± 0.00005 $^a$</td>
<td></td>
</tr>
<tr>
<td>Protocorm: Stage four</td>
<td>0.00107 ± 0.00017 $^b$</td>
<td></td>
</tr>
<tr>
<td>Protocorm: Stage five</td>
<td>0.04841 ± 0.01621</td>
<td>-</td>
</tr>
</tbody>
</table>
**Figure 5.8** a. Experimental set-up investigating the ability of fungal isolate (2.4.1, *T. calospora*) to access and supply inorganic phosphorus (orthophosphoric acid provided in an 8 mm disc of water agar (absent in picture) inserted into 10 mm well (white circle) to the developing *M. media* protocorm (stage five, white arrow). b. Digital autoradiograph of a similar petri-dish after scanning, highlighting the movement of $^{33}$P throughout the mycelial network (within the fungal compartment, black circle) and into the developing protocorm (within the plant compartment, black arrow). Red: higher levels of radioactivity, Blue: detectable levels of radioactivity.

**Figure 5.9** a. Experimental set-up investigating the ability of fungal isolate (2.4.1, *T. calospora*) to access and supply organic phosphorus (inositol polyphosphate provided in a disc of water agar, white circle) to the developing *M. media* protocorm (stage five, white arrow). b. Digital autoradiograph of the same petri-dish after scanning, highlighting the movement of $^{33}$P throughout the mycelial network (within the fungal compartment, black circle), and into the developing protocorm (within the plant compartment, black arrow). Red: higher levels of radioactivity, Blue: detectable levels of radioactivity.
5.3.6 Experiment 5.5 - Microcosm experiments involving the use of radioactively labelled inorganic and organic P

Experiment 5.5.1 - Capture and transfer of inorganic P

Digital autoradiographic scanning of microcosms (Figure 5.10 a,b) revealed the capture of P from the injected soil core, and its distribution through the mycelial network to the attached orchid. The image provides visual evidence of $^{33}$P accumulation in the orchid roots, and the transfer of P from the soil core to the distal parts of the network, where there is accumulation of radioactivity at the tips of the hyphal fans.

Autoradiographic analysis of microcosms with cores twisted at two day intervals revealed that even in these, low levels of P transfer into the surrounding agar could be detected. This provided evidence that despite ‘twisting’ of cores, some leakage of the isotope could occur from the injected soil into the surrounding agar. Such leakage is probably inevitable in systems of this kind, because, even in the presence of core twisting, physical connection between the agar and the soil within the core can be readily re-established over the two days ensuing after hyphal breakage.

The differences between and the relative importance of the direct hyphal pathway seen in the non-twisted systems and those with hyphal breakage are revealed by quantitative analysis of $^{33}$P accumulation in orchid tissues using liquid scintillation counting (Figure 5.11, Table 5.3). Low levels of P accumulation were detectable in orchid tissues in the twisted microcosm starting between harvest two and three. The apparently anomalous early appearance of low levels of radioactivity in the twisted microcosms, which is probably attributable to the occurrence of diffusion in some of these chambers, is fully considered in the discussion (Section 5.4.5). By Harvest six, highly significant differences were observed in the P accumulation of plants belonging to the two systems (Table 5.3).
Figure 5.10  
a. Harvest six microcosm (non-twisted soil core), showing the agar and orchid (white arrow) prior to imaging by digital auto-radiography. Soil core (black arrow) has been removed to avoid its quenching effects. b. Digital auto-radiograph of (a), highlighting the uptake and movement of $^{33}$P by mycorrhizal fungi, from soil core (black arrow) into the entire mycorrhizal network, and to orchid roots (black lined arrows). Although $^{33}$P was detected in the shoot by liquid scintillation counting, levels of radioactivity were insufficient for detection by digital auto-radiography. Red: higher levels of radioactivity, Blue: detectable levels of radioactivity.

Figure 5.11  
Accumulation $^{33}$P orthophosphate (pictograms, pg) by plants of *M. media* grown in microcosms either with hyphal connections between plants and soils intact (solid line, circles) or broken by twisting (dashed line, triangles). Three plants were harvested from each treatment every 2 days for 12 days. Each system was supplied with 0.5 MBq of $^{33}$P labelled orthophosphoric acid, containing 115 pg of labelled P.
**Table 5.3** Experimental coefficients of total $^{33}$P uptake (picograms, pg) in intact (non-twisted) and twisted microcosms after 12 days exposure to the isotope. The coefficients were calculated from a three-parameter log-logistical curve that was fitted onto a mean model for twisted and non-twisted mycorrhizal treatments across six harvests (total of 12 days). The coefficients represent the upper-limits ($d$) for P uptake in both treatments and show significant differences between the two treatments at a level of $P < 0.001 = ***$; or $P < 0.01 = **$, $n = 3$

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Upper Limit ($d$)</th>
<th>slope ($b$)</th>
<th>slope inception ($e$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-twisted</td>
<td>0.096 ± 0.003</td>
<td>-1.8 ± 3.9</td>
<td>4.94 ± 0.02</td>
</tr>
<tr>
<td>Twisted</td>
<td>0.032 ± 0.002</td>
<td>-1.6 ± 3.6</td>
<td>3.15 ± 0.35</td>
</tr>
</tbody>
</table>

**Experiment 5.5.2 - Capture and transfer of organic P**

Liquid scintillation counting of orchid tissue has revealed the assimilation of organic P by the mycorrhizal symbiont from the soil channel, and the subsequent transport of P into the host orchid (Table 5.4). This provides evidence that the mycorrhizal symbionts of *M. media* are able to improve the P nutrition of the orchid by accessing and utilising organic forms of P.

**Table 5.4** Mean contents of $^{33}$P (picograms, pg) in *M. media* adults and their respective tissue types (after 12 days exposure to $^{33}$P inositol polyphosphate), compared to $^{33}$P content of the control treatment. No statistical differences ($P > 0.05$) in $^{33}$P content were observed between the different tissue types.

<table>
<thead>
<tr>
<th>M. media</th>
<th>Exposed to $^{33}$P inositol polyphosphate</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult (overall)</td>
<td>0.00918 ± 0.00077</td>
<td>0.0013</td>
</tr>
<tr>
<td>Adult : Leaf</td>
<td>0.0057 ± 0.0013</td>
<td>0.00047</td>
</tr>
<tr>
<td>Adult: Tuber</td>
<td>0.0019 ± 0.0008</td>
<td>0.00072</td>
</tr>
<tr>
<td>Adult: Roots</td>
<td>0.0015 ± 0.0007</td>
<td>0.00005</td>
</tr>
</tbody>
</table>
5.4 Discussion

5.4.1 Experiment 5.1 - Phosphorus concentrations and contents of *M. media* tissue – baseline values

The tissues of the minute *M. media* seeds were shown to have a P concentration of approximately 3000 µg g\(^{-1}\) DW (≈ 0.3%). This is a high value relative to those seen in protocorms, or the differentiated organs of the adult plants. At the level of the individual whole seed, the weights of which are calculated (Chapter 2) to be approximately 0.826 µg per seed, the total P content is 0.0029 µg, equivalent to 0.35% of seed weight. Unfortunately, in the apparent absence of measurements of this kind in other orchid species, it is not possible to determine whether these values are representative of orchid seeds in the wider family. This can be easily rectified by some future work investigating the P contents of seeds produced by other sympatric orchids.

What is evident, however, is that since the P concentrations of the seed of *M. media* are elevated relative to those of the protocorms and adult organs, there may be selective provisioning of P to seed in the maturing capsule. Such a strategy could be important in enabling sustenance of the very earliest stages of germination, prior to the development of the symbiotic condition, and might help explain the ability to sustain the earliest stages of germination on water agar.

However, when compared with the P concentrations determined for non-orchid species that are likely to be co-associates of *M. media* under natural bushland conditions, the orchid P concentrations are relatively low. In frequently co-occurring *Banksia* species, mean seed P concentrations of 10880 µg g\(^{-1}\) DW are approximately three times greater than those of the orchid (Denton *et al.*, 2007). It has been suggested that such high P concentrations in seeds of the Proteaceae may represent a crucial adaptation improving the survival and early development of members of this family in soils characteristically low in P content (Pate & Dell, 1984; Lamont & Groom, 2002).

In the absence of roots throughout the process of protocorm development, the progressive increase in baseline P contents of *M. media* tissues must be attributable to
P gained from their fungal symbionts. In the oatmeal agar based systems used for symbiont driven protocorm cultivation (utilised in this study), the ultimate source of P gain by the orchid must be the agar itself. In nature, it must be soil, where both inorganic and organic sources of the element are potential sources for fungal exploitation.

The P concentrations of the adult tissues of the orchid, averaged over three tissue types (leaf, root and tuber) are at 1725 µg g⁻¹ DW, considerably higher than those of its frequent co-associates in the field, which have been reported to be around 990 µg g⁻¹ DW (Wright et al., 2004). Interestingly, in this connection, the *M. media* leaf P concentration of 2096 µg g⁻¹ DW was approximately ten times higher than those reported for Banksia species (= 200 µg g⁻¹ DW). The orchid values are more similar to the = 2000 µg g⁻¹ DW reported for crop species (Kuo et al., 1982; Epstein & Bloom, 2005; Denton et al., 2007; Marschner & Marschner, 2012). The occurrence of such high tissue P concentrations in an orchid growing in soils characterised by their extreme P limitation, is indicative of the likelihood that its mycorrhizas provide an effective P scavenging mechanism in these plants.

5.4.2 Experiment 5.2.1 - Plant growth responses to P treatments

Although both organic and inorganic forms of phosphorus exist within soil, studies have suggested that the inorganic form of phosphorus is the preferential form utilised by plants (Olsen, 1954; Pate & Dell, 1984; Epstein & Bloom, 2005; Smith & Read, 2008; Marschner & Marschner, 2012). Thus, it was important to determine if there was any preferential utilisation of these different forms of P in the case of *M. media*, and if there was, what was the preferred form.

The experimental analysis of the ability of mycorrhizal protocorms to acquire P in agar systems showed that in the case of inorganic P, provisioning of the protocorms increased with increasing content of the element in the growth medium. The main effect was observed between zero P provision, and 2.00 ppm, with P concentrations/contents of protocorms increasing with harvests. This provides experimental confirmation of the ability of the mycorrhizal fungus to capture inorganic P from the media. Previous studies have shown that orchid mycorrhizal fungi, whether grown in pure culture (Nurfadilah et al., 2013), or in symbiosis (Smith, 1966; Alexander
et al., 1984; Cameron et al., 2006), can capture P in this form, but have not quantified plant responses over a concentration range. The small increases in P contents of protocorms over time in the zero ppm inorganic P treatment may also confirm the ability of the symbiont to scavenge for P contained within the agar gel, while at the same time suggesting that the availability of this ‘background’ P was insufficient to enable optimal protocorm growth. Clearly the P demands of such small protocorms are themselves small and it appears that the provision of 2 ppm of additional ‘free’ P is sufficient to enable the enhancement of P acquisition.

Increasing concentrations of organic P had relatively little impact upon protocorm P content. This could reflect either the fact that this form of P was unavailable to the fungus, or that the fungal symbiont preferentially used the carbon-based form of the element to sustain its own development, rather than to provision the protocorm. Some evidence for this possibility was provided by patterns of P acquisition, revealed in the auto-radiographic analyses of organic P uptake and distribution (Figure 5.9 b). The ability to take up organic P would be predicted since a significant proportion of the P in bushland soils is likely to be retained in organic form (McArthur, 1991). In pure culture experiments, Nurfadilah et al (2013) demonstrated the ability of a number of orchid mycorrhizal fungi to utilise both organic and inorganic P, but the levels of both forms of the element that were supplied in their experiments were unrealistically high.

The amounts of P in agar have generally been assumed to be either so low, or sufficiently inaccessible to be of no consequence in studies of plant, or indeed fungal growth. However, in very small plants or plant propagules such as protocorms, the presence of P as a natural constituent of the alginates used in the gelling process should be taken into account. The ability to capture such P from water agar is evident from the small but albeit detectable increase in protocorm P contents in the agar cultures to which no supplementary P was added (Figure 5.3).

5.4.3 Experiment 5.2.2 - Phosphorus treatments – fungal growth responses

While the organic form of added P yielded the greatest extension growth in T. calospora (isolate 2.4.1) emerging from protocorms, this P response was not repeated in terms either of higher seedling P concentrations, or greater protocorm yields. The inherent presence of an unquantified residual P content, probably of both inorganic
and organic types, in the agar makes it very difficult to obtain accurate interpretation of results. Future studies both of fungal growth and protocorm growth responses should preferably be carried out in media lacking in ‘background’ amounts of P.

5.4.4 Experiment 5.3 Nitrogen treatments – plant growth responses

Nitrogen in the form of ammonium has been suggested to be better at stimulating phosphorus uptake when compared to nitrate, and these differences have been largely attributed to the differences in pH around the rhizosphere resulting from nitrogen uptake (Arnon, 1939; Olson & Dreier, 1956; Grunes, 1959; Leonce & Miller, 1966; Riley & Barber, 1969; Riley & Barber, 1971; Marschner & Marschner, 2012). By altering rhizosphere pH to varying degrees, the solubility of phosphorus (within substrate) is affected, which in turn, influences the amount of phosphorus available for plant uptake.

Similar results were observed from this study, with the ammonium (ammonium sulphate) treatment generating seedlings with the highest phosphorus concentrations (Figure 5.6 c). While the 20 ppm ammonium treatment yielded seedlings at harvest 4 with the highest phosphorus concentrations (1251.4 µg g⁻¹ DW), any further increase in substrate ammonium levels (40, 60, 80 and 100 ppm) resulted in the overall decrease of seedling phosphorus concentrations. This infers that although ammonium was able to promote phosphorus accumulation, peak efficiency occurred at 20 ppm (of ammonium), and that any additional increases in substrate ammonium concentrations resulted in decreased phosphorus accumulation within M. media seedlings.

Phosphorus concentrations of M. media seedlings subjected to the two nitrate treatments (Figure 5.6 a and b) were relatively constant across all the concentrations. This indicates that although nitrates were unable to promote phosphorus uptake as efficiently as ammonium, it still influenced the uptake of phosphorus and that there were no specific nitrate concentrations which promoted phosphorus accumulation. Interestingly, at harvest four, the phosphorus concentrations of seedlings exposed to the organic nitrogen treatment (glutamine) were generally on the high side, and as such, suggest the need to raise the possibility that response of the fungi to progressively increasing quantities of glutamine may have been influenced by the
inherent parallel increase in carbon supply. Mycorrhizal fungi are known (as pointed out in Chapter 2) to become parasitic in the presence of labile exogenous C.

5.4.5 Experiment 5.4 and 5.5 - Experiments (petri-dish and microcosm) involving the use of radioactively labelled inorganic and organic phosphorus

The uptake of $^{33}\text{P}$ labelled orthophosphoric acid and inositol polyphosphate by $T.\ calospora$, and the subsequent movement of $^{33}\text{P}$ into the mycorrhizal network and developing $M.\ media$ protocorms, combined with the lack of $^{33}\text{P}$ within $M.\ media$ protocorms of the control treatments, confirms that mycorrhizal fungi supply P to $M.\ media$. The results of the current study thus also confirm the findings of Smith (1966), who described the uptake of $^{32}\text{P}$ orthophosphate by $Rhizoctonia\ repens$ Bernard (= $T.\ calospora$) and the subsequent transfer of labelled P into developing protocorms of the northern hemisphere terrestrial orchid, $Dactylorhiza\ purpurella$ Soo.

Results from the microcosm experiments yielded two main conclusions: mycorrhizal fungi (mostly $Tulasnella$ spp.) being able to access inorganic and organic forms of phosphorus applied to habitat soil, and that hyphal breakage significantly impacted on $M.\ media$ (adult) phosphorus accumulation. Mycorrhizal symbionts isolated from adult $M.\ media$ (microcosm experiments) were identified in most cases as being $Tulasnella$ spp. (see Appendix 1 for details), which confirms the earlier (see Chapter 4) finding that this is the most widely occurring fungal symbiont of $M.\ media$.

The ability of $T.\ calospora$ to take up and transfer $^{33}\text{P}$ through its mycelial network to seedlings of the orchid, is reminiscent of the same capabilities demonstrated indirectly by Alexander et al (1984) and directly by Cameron et al (2006), though both studies used a different study system – the orchid $Goodyera\ repens$ (R.Br) and fungal symbiont $Ceratobasidium\ cornigerum$ (D.P Rogers). A lag period between isotope application and isotope accumulation within orchid (~4 days for $M.\ media$) was also reported by Alexander et al (1966), who suggested that it was a consequence of the time required for the uptake and transport of labelled phosphorus within the mycorrhizal network.

The relatively low $^{33}\text{P}$ concentrations observed within $M.\ media$ shoots may be attributable to the short experimental duration. The presence of $^{33}\text{P}$ in $M.\ media$ shoots, within twelve days of isotope application, suggests that active transportation
of $^{33}$P (from root to shoot) was occurring, and that one would expect higher shoot $^{33}$P concentrations if future experiments were conducted for longer periods. Although significant amounts of $^{33}$P were detected in the roots of *M. media*, transport of $^{33}$P within the mycorrhizal network was not exclusively to the roots (Figure 5.10 b), with $^{33}$P being detected throughout the entire mycorrhizal network. It was evident from the autoradiographs that the mycobiont was assimilating and transporting $^{33}$P of both organic and inorganic types, so any failure to transfer the element to the associated plants can probably be best explained by retention with the tissues of the fungus itself. The movement of $^{33}$P within the mycorrhizal network could potentially be explained by the mycorrhizal symbiont utilising $^{33}$P for its own growth.

The microcosm experiments also highlighted the impact of hyphal breakage on *M. media* phosphorus accumulation, confirming the importance of an intact mycorrhizal network for the provision of phosphorus in *M. media*. At the conclusion of the experiment, *M. media* plants with intact hyphae had $^{33}$P concentrations (0.096 ± 0.003 pg) that were almost three times those of plants with severed hyphae (0.032 ± 0.002 pg, Table 5.3). A similar effect of hyphal breakage on phosphorus accumulation within plants associating with arbuscular mycorrhizal fungi was reported in an earlier study (Johnson *et al.*, 2001), where the authors found shoot $^{33}$P concentrations of plants with intact hyphae to be ten times higher than the shoot $^{33}$P concentrations of plants with severed hyphae.

It is evident from the results presented in Figure 5.11, that radioactivity, albeit at extremely low levels, appears first (at harvest three) in plants of the twisted series. While this low level of activity reaches a plateau that is maintained through the remainder of the harvests in this treatment, there is a rapid and accumulating level of P in plants of the non-twisted systems, from harvest four to harvest six. The cause of the apparently anomalous appearance of radioactivity in plants grown in the twisted cores must be considered. It appears most likely that this is attributable to diffusion of activity from the soil cores into the agar surrounding them. While twisting of the cores certainly broke all hyphal connections, the physical agar to core wall connections would be re-joined on every occasion after twisting, so providing the possibility for a diffusional pathway. These systems contrasted with those employed in pioneering experiments developing these techniques (Johnson *et al.*, 2001), which involved soil to
soil connections, rather than agar to soil. Building on the results of this investigation, future studies should be carried out on soil systems in order to better understand what happens in nature.

The petri-dish experiments used in the parallel studies with protocorms provided a clear break in the potential agar diffusion pathway by maintaining an agar-free channel and also provided a plastic central barrier. That is that diffusion is not the major route whereby $^{33}$P reaches the plants in the twisted systems is evidenced by the fact that the pathway of transfer is clearly revealed in the autoradiographs to be the mycelial network.
5.5 Conclusions

Results of this chapter have improved the understanding of phosphorus nutrition in M. media, and have elucidated certain characteristics which are likely to have contributed to the ruderal habit of the species. Specific observations include:

- Elevated concentrations of phosphorus within M. media seed
  - This ‘pre-loading’ of seeds with phosphorus can be viewed as an adaptation of the species to maximise offspring survival.
- Fungal symbionts (of M. media utilised in microcosm experiments) were mostly confirmed as Tulasnella species.
  - Supports the view that M. media predominantly associates with Tulasnella (Chapter 4).
- That these mycorrhizal symbionts are able to assimilate and transfer both organic and inorganic forms of phosphorus to M. media.
  - Although there may be a reduced ability to assimilate and transfer organic phosphorus, but this could arise through competition between the symbionts for the organic resource.
  - Results also suggest that these mycorrhizal symbionts are disturbance tolerant.

These observations provide support for HYPOTHESIS 4: That the mycorrhizal fungi play a central role in the provision of the growth-limiting element, Phosphorus, to the developing orchid. This chapter has demonstrated the central role played by the mycorrhizal symbionts in facilitating the movement of phosphorus to M. media. The next chapter will investigate this relationship further by experimentally analysing the carbon economies of the relationship between M. media and its mycorrhizal symbiont at both the protocorm and adult stages of development.
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Chapter 6: Carbon nutrition of *M. media*
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6. Abstract

Although the provision of carbon by the mycorrhizal symbiont is generally assumed to be critical for the successful germination of orchid seeds, this has only been demonstrated in a few orchid species. Following on from the previous phosphorus investigations, this following chapter investigates the carbon nutrition of *M. media*. The ability of *Tulasnella calospora* (fungal isolate 2.4.1), a widely occurring fungal symbiont of *M. media*, to mobilise carbon (C) from $^{14}$C labelled cellulose has been demonstrated. The C liberated from this polymer was assimilated from the cellulose embedded within the agar matrix, and transferred through the mycelial network of the fungus, and into developing *M. media* protocorms which it had colonised earlier. This confirms the presence of an ‘up-flow’ of C in the *M. media* mycorrhizal system. Since cellulose is acknowledged to be amongst the most abundant of all naturally occurring sources of C within soil, this observation confirms that mycorrhizal symbionts have the potential to play key roles in facilitating the development of *M. media* protocorms in nature.

In addition, experiments using adult plants, the shoots of which were supplied with $^{14}$CO$_2$ in sealed transparent containers, have shown that carbon fixed by the photosynthesising plants were being transferred below-ground to roots, and outwards into the soil-borne mycelial network of *T. calospora*. Thus, in addition to the ‘up-flow’ pathway in heterotrophic protocorms, there is a ‘down-flow’ of C in the autotrophic adult phase.

It was also demonstrated that carbon emerging into the fungal network along the ‘down-flow’ pathway could be allocated directly into protocorms of *M. media*, which have colonised the agar surface adjacent to the adult roots. This important observation indicates the potential for ‘parental nurture’ of the next orchid generation.

All of the abovementioned observations suggest that there is a robust and dynamic movement of carbon within *M. media* ‘communities’, and that more investigations will need to be carried out in order to fully characterise it.
6.1 Introduction

Seeds of the Orchidaceae, most of which consist only of undifferentiated embryo contained within a thin testae, have long been assumed to contain insufficient nutrients to allow for independent germination (Rasmussen, 1995; Arditti & Ghani, 2000). As such, it is generally accepted that orchids are myco-heterotrophic (completely dependent on their mycorrhizal fungi for the provision of essential nutrients such as carbon), at least during the early stages of their life cycle (Arditti, 1967; Leake, 1994; Rasmussen, 1995; Smith & Read, 2008).

While most orchid germination studies have reported significant increases in both orchid seed germination and protocorm growth when seeds are co-cultured with appropriate mycorrhizal fungi, the rationale behind these so called ‘benefits’ remained unresolved until 1966. Smith (1966) conclusively demonstrated the assimilation and movement of $^{14}$C, from mycorrhizal symbiont (Rhizoctonia repens = T. calospora) into developing non-photosynthetic orchid (Dactylorchis purpurella = Dactylorhiza purpurella), and firmly established the importance of mycorrhizal fungi in the provision of carbon to germinating orchid seeds. However, after this initial study, the field of orchid seed nutrition was largely neglected, and it was not until recently, that the movement of nitrogen, in addition to carbon, was tracked from assimilation by mycorrhizal symbiont, into developing non-photosynthetic protocorms of Spiranthes sinensis Pers. (Kuga et al., 2014).

With the advent of both radioisotope and stable isotope studies, the partially myco-heterotrophic (deriving carbon by both self-assimilation, and from its fungal symbiont(s)) nature of numerous terrestrial orchids has been elucidated (Gebauer & Meyer, 2003; Bidartondo et al., 2004; Julou et al., 2005; Cameron et al., 2006; Cameron et al., 2007; Cameron et al., 2008; Látalová & Baláž, 2010; Liebel et al., 2010; Motomura et al., 2010; Yagame et al., 2012). Results have suggested that between 30 to 50 % of the carbon requirements of these orchids were being fulfilled by their fungal symbionts, although sink strengths (for carbon) were not fixed (Julou et al., 2005; Látalová & Baláž, 2010; Motomura et al., 2010; Girlanda et al., 2011; Yagame et al., 2012). Levels of irradiance (of the orchid habitat) have been suggested as a major factor influencing the amount of carbon supplied by the mycorrhizal symbiont, as
studies have shown larger amounts of fungal derived carbon being supplied to orchids growing under deep shade within forests, while orchids growing in open habitats were found to derive little or no carbon from their fungal symbionts (Gebauer & Meyer, 2003; Bidartondo et al., 2004; Julou et al., 2005; Liebel et al., 2010).

While the flow of carbon and other nutrients is widely accepted to occur unidirectionally, from mycorrhizal fungi to orchid (Burgeff, 1959; Hadley & Purves, 1974; Alexander & Hadley, 1985; Rasmussen, 1995; Jones & Smith, 2004; Rasmussen & Rasmussen, 2009), the notion of nutrients, specifically carbon, moving in the opposite direction, from orchid to mycorrhizal symbiont, has been a point of some contention.

The possibility of a mutualistic relationship between orchids and their mycorrhizal symbionts, where plant photosynthate is exchanged for fungal derived nutrients (such as nitrogen or phosphorus), as demonstrated in arbuscular and ectomycorrhizal symbioses, remains largely neglected, despite the fact that some recent studies have highlighted the potentially mutualistic nature of orchid mycorrhizal symbioses. In addition to the studies carried out by Cameron et al (2006 and 2008), which have conclusively demonstrated the mutualistic flow of nutrients between Goodyera repens, and its mycorrhizal symbiont Ceratobasidium cornigerum, Liebel et al (2010) has suggested that the net transfer of carbon, from orchid to mycorrhizal symbiont, was also occurring in approximately 22 orchid species found within the Mediterranean region and Macaronesia.

Despite the fact that the orchid of this study, Microtis media, has been aggressively expanding into new and novel habitats (such as horticultural beds) in the past decade (K. Dixon, pers comm), relatively little is known about the factors contributing to the recruitment success of the species. Although seedling recruitment has been suggested as one of the main constraints on orchid populations (McCormick & Jacquemyn, 2014), this constraint appears to be less important in M. media given its ruderal expansion. Despite that, orchid recruitment has been suggested to be heavily influenced by the presence of adult orchids, with these adults either reflecting suitable microsites for growth, or the presence of suitable mycorrhizal symbionts (Batty et al., 2001; Diez, 2007; Jacquemyn et al., 2007; Jacquemyn et al., 2012). Given that recruitment of M. media in both field and glasshouse conditions has been observed to occur mainly in the vicinity of adults (Figure 6.1), it can be confidently assumed that the presence of
adult *M. media* plants impact positively on *M. media* recruitment. However, the underlying basis for this positive impact on *M. media* recruitment remains unresolved, and will be investigated in this chapter.

![Figure 6.1](image)

**Figure 6.1.** a. Typical colonization of pots within the Kings Park living collection, by *M. media* (green shoots). b. New germinants of *M. media* (white arrows), occurring along the roots (red arrows) of pre-existing *M. media* plants.

### 6.1.1 Aims

Given that the nature (mutualistic or parasitic) of the relationship between *M. media* and its mycorrhizal symbiont(s) remains largely unknown, this chapter aims to conduct preliminary investigations which would determine the physiological basis of this relationship. Specifically, the following questions were asked:

- **Is the mycorrhizal symbiont supplying carbon to developing non-photosynthetic *M. media* protocorms?**
  - To determine if there is ‘up-flow’ of carbon from fungi to orchid.

- **Is there flow of carbon (in terms of orchid photosynthate) occurring from green *M. media* plants to its mycorrhizal symbiont(s)?**
  - To determine if there is ‘down-flow’ of carbon from orchid to fungi.

- **Can photo-synthetically derived carbon, supplied by green *M. media* plants to their mycorrhizal symbiont(s), be transferred into developing non-photosynthetic protocorms of *M. media*?**
  - To determine if ‘parental nurture’, or inter-generational sharing of carbon was occurring within *M. media*. 

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6.2 Materials and Methods

6.2.1 Investigations into the ability of fungal isolate 2.4.1 (*T. calospora*) to utilise \(^{14}\text{C}\) labelled cellulose as a carbon source, and the extent to which this carbon is subsequently transferred into developing *M. media* protocorms

Non-photosynthetic stage three \((n = 15)\) and four \((n = 15)\) *M. media* protocorms were generated symbiotically with fungal isolate 2.4.1 (*T. calospora*) as per conditions outlined in Chapter 3. Individual protocorms were transferred into split petri-dishes (containing water agar, \(6 \text{ g}^{-1}\) agar and 1 litre of distilled water), with the compartment containing the protocorm henceforth known as the plant compartment. These dishes were incubated in total darkness (achieved by wrapping dishes in two layers of aluminium foil) at 15 °C for ten days to allow for the mycorrhizal network to develop over the plastic barrier, and to fully colonise the fungal compartment (not containing the protocorm). A 10 mm disc of agar was then removed from the fungal compartment using a cork borer, and replaced with an 8 mm disc of water agar (containing 3.77 µg of \(^{14}\text{C}\)) supplemented with \(^{14}\text{C}\) labelled cellulose (Figure 6.2 a). An additional 15 stage four protocorms were utilised as controls, in which a 5 mm strip of agar was removed from the fungal compartment (distal to plastic barrier, Figure 6.2 b) before the introduction of \(^{14}\text{C}\) cellulose supplemented agar disc. Labelled dishes were incubated for an additional ten days before imaging by digital auto-radiography (Packard Instant Imager, Isotech, Chesterfield, UK) and analysed for their \(^{14}\text{C}\) contents.

Agar and protocorms were freeze dried for five days before recording their respective dry weights. The \(^{14}\text{C}\) contents of these samples were determined by oxidising (Model 307, Packard Sample Oxidiser, Isotech, Chesterfield, UK), and trapping the released \(^{14}\text{CO}_2\) in 10 ml of Carbo-sorb (Perkin Elmer, Massachusetts, USA). This was followed by the addition of 10 ml of Permafluor scintillation cocktail (Perkin Elmer, Massachusetts, USA), before samples were measured for their radioactive activity using liquid scintillation counting (Packard Tri-carb 3100 TR, Isotech, Chesterfield, UK). The \(^{14}\text{C}\) contents of protocorms were calculated as per Cameron *et al* (2008).
6.2.2 Investigations into the flow of carbon from *M. media* to its mycorrhizal symbiont

Eight week old photosynthetic *M. media* seedlings (n = 15) grown with fungal isolate 2.4.1 (*T. calospora*) were utilised to investigate the movement of carbon from orchid into the mycorrhizal network. Individual seedlings were transferred into 90 mm sterile petri-dishes (containing water agar), and incubated under light conditions at 15 °C for seven days to allow for the development of the mycorrhizal network (Figure 6.6 a).

Following incubation, lids of these petri-dishes were removed before they were placed into an airtight labelling chamber (polythene bag), together with an Eppendorf tube containing 1.85 MBq (5 µl) of 14C labelled sodium bicarbonate solution (Perkin Elmer, Massachusetts, USA). The labelling chamber was then heat sealed to ensure that it was air tight, before the release of 14CO2 was facilitated with the injection of 1.5 ml of 25% (v/v) lactic acid solution into the Eppendorf tube. The injection port was sealed with airtight polyethylene tape. *Microtis media* seedlings were exposed to 14CO2 for 48 hours before these systems were imaged using digital auto-radiography (Packard Instant Imager, Isotech, Chesterfield, UK). No analyses was carried out on the 14C contents of *M. media* seedlings and their corresponding fungal networks as this was essentially an investigation to determine if any carbon flow occurred from orchid to mycorrhizal symbiont.
6.2.3 Investigation of the potential for ‘parental nurture’ of *M. media* protocorms

Following the establishment of carbon flow from photosynthetic *M. media* seedlings into their mycorrhizal networks, the next aim of this investigation was to determine if this photo-synthetically derived carbon (supplied to the mycorrhizal symbiont by the orchid) could be transferred into developing non-photosynthetic *M. media* protocorms.

Dormant *M. media* tubers (*n* = 5), together with habitat soil, were removed from bushland within Kings Park in the summer of 2013. Tubers were potted up in habitat soil, and revived under glasshouse conditions in 2014, following the onset of autumn. These adult *M. media* plants were allowed to grow for six weeks (still in a state of active shoot growth) before being removed from the pots, cleaned (roots cleaned of any soil particles under tap water) and introduced individually into microcosms (roots secured with cooled sterile water agar, Figure 6.3).

Microcosms comprised of 25 cm sterile square petri-dishes filled with water agar. The dishes had a notch cut in one vertical wall through which the green shoot (Figure 6.3, arrow) of the orchid could protrude into the light. After introduction of adult plants, microcosms were incubated under light conditions at 15 °C for two weeks in order to allow for the development of the mycorrhizal network, before unsterilized *M. media* seeds (approximately 200 at each location) were introduced at five different locations on the mycorrhizal network (Figure 6.3, circles). Given that the roots of the *M. media* plants were not surface sterilized before introduction into the microcosms, the decision to introduce unsterilized *M. media* seeds (as opposed to sterilized *M. media* seeds) onto the mycorrhizal networks of these plants was made in an attempt to mimic nature.

Individual microcosms, with the exception of the protruding shoots, were then wrapped up in two layers of aluminium foil, in order to prevent any light exposure on the germinating *M. media* seeds. Although no photo-inhibition has been observed in germinating *M. media* seeds (see Chapter 2), the rationale behind keeping the developing protocorms in complete darkness was to ensure that they remained non-photosynthetic. These germinating seeds were visually assessed on a weekly basis for their development, and labelling was carried out after these seeds developed into
stage three protocorms. One microcosm was designated as the control treatment, which involved the removal of the entire *M. media* plant (but not the protocorms) just prior to labelling with $^{14}$CO$_2$.

The labelling of these microcosms with $^{14}$CO$_2$ was conducted in a similar fashion to that described in section 6.2.2. Shoots were exposed to $^{14}$CO$_2$ for 48 hours, after which the labelling chamber was vented of all $^{14}$CO$_2$ by opening it within a fume cabinet. Unfortunately, due to time limitations, only one microcosm and the control could be imaged (by digital auto-radiography) and analysed for their $^{14}$C contents. Both plant and agar had to be lifted out of the microcosms and laid onto specially designed Perspex sheets (Figure 6.8 a) before imaging by digital auto-radiography could be undertaken. Only plant material was analysed for their $^{14}$C contents (as per conditions highlighted in Section 6.2.2), with approximately 100 protocorms sampled from each location (total of 500 protocorms). The $^{14}$C contents of plant material were calculated as per Cameron *et al* (2008).

![Figure 6.3. Microcosm utilised in the investigation of carbon flow from adult *M. media* (black arrow) into developing *M. media* protocorms (position highlighted by black circles). Following the introduction of adult plants, microcosms were incubated for two weeks before unsterilized *M. media* seeds were introduced onto the mycelial network. Labelling of microcosm with $^{14}$CO$_2$ was only conducted when seeds had developed into stage three protocorms.](image)
6.3 Results

6.3.1 Investigations into the ability of the mycorrhizal symbiont to utilise cellulose as a carbon source and of the extent to which this carbon is subsequently transferred into developing *M. media* protocorms

The ability of fungal isolate 2.4.1 (*T. calospora*) to utilise cellulose (14C labelled cellulose) as a carbon source has been demonstrated (Figures 6.4 and 6.5). Figure 6.4 (b) highlights the accumulation of 14C in both stage three (Figure 6.5, 0.00099 ± 0.00030 µg) and four (0.00361 ± 0.00011 µg) *M. media* protocorms, although statistical differences were not detected (U = 40.0, P = 0.494). However, statistical differences (U= 4.5, P = 0.029) were detected between the 14C contents of stage four protocorms, and the 14C contents of stage four protocorms subjected to the control treatment (0.00001 ± 0.00000 µg). In addition, statistical differences were also detected in the 14C contents of stage four protocorms, and their corresponding fungi (0.02760 ± 0.00072 µg, t= -3.209, P = 0.019, Figure 6.4 b and c).

![Figure 6.4.](image)

Figure 6.4. a. The amount of 14C supplied, and (b) mean content (± SE) of 14C within *M. media* protocorms of different developmental stages (S3 – stage three, S4 – stage four, S4 (C) – stage four controls). c. Mean 14C content (± SE) of mycorrhizal network emanating from stage four *M. media* protocorms. Note the differences in scale between a, b and c.
Figure 6.5. a. Experimental set up investigating the movement of labelled carbon from source point (agar disc containing $^{14}$C labelled cellulose, black circle) to a stage three *M. media* protocorm (black arrow). b. Digital auto-radiograph (48 hour exposure) of a similar petri-dish, showing the movement of $^{14}$C out from source point (agar disc, black circle), into the mycorrhizal network and *M. media* protocorm (black arrow). The colour scale bar represents the number of counts detected in pixel areas (0.25 mm$^2$) over a period of 48 hours.
6.3.2 Investigations into the flow of carbon from \textit{M. media} to its mycorrhizal symbiont

The flow of carbon (fixed by photosynthesis), from green \textit{M. media} to its mycorrhizal symbiont has been demonstrated. Within 48 hours of exposure to $^{14}\text{CO}_2$, carbon fixed by \textit{M. media} (through photosynthesis) was shown to be transferred into the mycorrhizal symbiont (Figure 6.6) by way of the connection between the orchid and external mycelial network. The movement of $^{14}\text{C}$ occurred throughout the mycorrhizal network, with elevated concentrations observed at the edges of the mycorrhizal network (Figure 6.6, b), where the actively growing hyphal tips were located. In addition, the direct uptake of $^{14}\text{CO}_2$ by the mycorrhizal symbiont can be confidently ruled out, as no radioactivity was detected in any of the control systems (\textit{M. media} plants removed just prior to exposure to $^{14}\text{CO}_2$).
Figure 6.6. a. Experimental setup of $^{14}$CO$_2$ labelling experiments. *Microtis media* seedlings were transferred into petri-dishes containing water agar, and incubated for seven days before labelling with $^{14}$CO$_2$ (1.48 MBq). b. Digital auto-radiograph (48 hour exposure) of the mycorrhizal network after labelling, showing the transport of $^{14}$C from *M. media* seedling to the actively growing mycorrhizal network. Accumulation of $^{14}$C was observed at the edges of the mycorrhizal network, as indicated by the predominately red edges of the mycorrhizal network. Colour scale bar represents the number of counts (of radioactivity) detected in pixel areas (0.25 mm$^2$) over a period of 48 hours.
6.3.3 Investigation of the potential for ‘parental nurture’ of *M. media* protocorms

Carbon, in the form of orchid photosynthate, has been determined as a source of carbon which could be utilised to fulfil the carbon requirements of developing non-photosynthetic *M. media* protocorms sharing the same hyphal network (Figures 6.7 and 6.8). The fixing of labelled \(^{14}\text{CO}_2\) by photosynthesizing *M. media* plants, and transfer of \(^{14}\text{C}\) labelled photosynthate into the mycelial network has been demonstrated, together with the subsequent movement of \(^{14}\text{C}\) from mycorrhizal network into developing non-photosynthetic *M. media* protocorms (Figure 6.8). Accumulation of \(^{14}\text{C}\) within these non-photosynthetic protocorms has been confirmed by both liquid scintillation counting (Figure 6.7 c) and digital autoradiography (Figure 6.8). Minute, almost undetectable amounts of \(^{14}\text{C}\) (0.000009 ± 0.000000 µg) were detected in protocorms of the control treatment, but these were not significantly above the measured background levels.

**Figure 6.7.** a. The amount of \(^{14}\text{C}\) supplied, and (b) overall \(^{14}\text{C}\) content of the *M. media* adult and the distribution of \(^{14}\text{C}\) between the shoot, roots and tuber. (c) Mean \(^{14}\text{C}\) content (± SE) of stage three non-photosynthetic *M. media* protocorms. Note the differences in scale within a, b and c.
Figure 6.8. a. Experimental setup investigating the occurrence of parental nurture, where $^{14}$C is transferred from photosynthetic *M. media* (black arrow), into developing *M. media* protocorms. b. Digital auto-radio-graph (24 hour exposure) of microcosm after 24 hours of exposure, showing the uptake of $^{14}$CO$_2$ by *M. media*, and subsequent movement of $^{14}$C into developing *M. media* protocorms (black circles), and into the mycorrhizal network (accumulated at the edges of the microcosm). c. Digital auto-radiograph of the same microcosm, after being exposed for 48 hours, confirming the presence of $^{14}$C within developing *M. media* protocorms. Colour scale bar represents the number of counts (of radioactivity) detected in pixel areas (0.25 mm$^2$) over a period of 48 hours.
6.4 Discussion

6.4.1 ‘Up-flow’ of carbon from mycorrhizal symbiont to developing *M. media* protocorms

Cellulose, a complex polysaccharide comprised of glucose subunits, is one of the main components contributing to the structural integrity of plants (Marschner & Marschner, 2012). Given its ubiquitous presence (within leaf litter and dead plant material), cellulose has been suggested as a major source of carbon for organisms (specifically fungi) existing within soil (Smith, 1966; Alexander & Hadley, 1985; Smith & Read, 2008; Nurfadilah et al., 2013). Given that members of the Tulasnellaceae have been reported to exist both as plant symbionts and saprophytes (McCormick et al., 2004; Garcia et al., 2006; Suárez et al., 2006; Smith & Read, 2008; Látalová & Baláž, 2010; McCormick et al., 2012; Ogura-Tsujita et al., 2012; Tan et al., 2014), it is without surprise that this investigation has established the ability of fungal isolate 2.4.1 (*T. calospora*) to utilise cellulose as a carbon source, as shown by the assimilation and movement of $^{14}$C (from $^{14}$C labelled cellulose) within the mycorrhizal network.

In addition, the subsequent movement of $^{14}$C from cellulose into developing non-photosynthetic *M. media* protocorms also conclusively demonstrates that mycorrhizal fungi are indeed responsible for the provision of carbon to developing *M. media* protocorms. Results from this investigation correspond with the observations of Smith (1966), who reported the assimilation and subsequent translocation of $^{14}$C ($^{14}$C labelled glucose) from mycorrhizal symbiont (*Rhizoctonia repens* = *T. calospora*) into developing non-photosynthetic protocorms of *Dactylorchis purpurella* (= *Dactylorhiza purpurella*). Similarly, the assimilation and translocation of $^{13}$C (from $^{13}$C labelled glucose) from mycorrhizal symbiont (*Ceratobasidium* sp.) to non-photosynthetic protocorms has also been recently reported to occur in *Spiranthes sinensis* (Kuga et al., 2014). Although $^{14}$C was detected within protocorms of the control treatment, in which all fungal connections (between the plant and fungal chambers) were severed prior to introduction of the isotope, levels of radioactivity detected were below background radiation levels.
Based on the results of this current study, which has highlighted the ability of *Tulasnella calospora* to utilise cellulose as a carbon source, and to translocate this carbon resource into developing *M. media* protocorms, the predominant association of *M. media* with *Tulasnella* spp. (see Chapter 4) can be viewed as a major adaptation which has contributed to the ruderal expansion (mostly into disturbed habitats inherently rich in cellulose such as mulched garden or horticultural beds) of the species. However, given that this investigation only looked at the provision of carbon for non-photosynthetic *M. media* protocorms, future studies utilising photosynthetic *M. media* plants will need to be conducted in order to positively establish the fully autotrophic or partially myco-heterotrophic nature of green *M. media* plants, and to determine if cellulose represented a major source of carbon for *M. media* under natural environments.

6.4.2 ‘Down-flow’ of carbon from photosynthetic *M. media* to mycorrhizal symbiont

Mycorrhizal symbiosis, have mostly been described (arbuscular and ectomycorrhizal systems) as mutualistic, where host plant derived carbon (photosynthate) is exchanged for fungal derived nutrients such as phosphorus and nitrogen (Smith & Read, 2008). However, in the field of orchid mycorrhiza, given that early investigations found no movement of carbon from orchid to mycorrhizal symbiont (Burgeff, 1959; Hadley & Purves, 1974; Alexander & Hadley, 1985), the nature of the orchid-mycorrhizal symbiont relationship has generally been assumed to be parasitic, in which the orchid stands to benefit solely from the uni-directional flow of nutrients from fungi to orchid (Rasmussen, 1995; Brundrett, 2004; Jones & Smith, 2004; Rasmussen & Rasmussen, 2009).

Surprisingly, this view is still accepted in some quarters, despite the fact that studies have highlighted the potentially mutualistic nature of the orchid-mycorrhizal symbiont relationship (Cameron *et al.*, 2006; Cameron *et al.*, 2007; Cameron *et al.*, 2008; Liebel *et al.*, 2010). Based on the results from this investigation, in which the movement of $^{14}$C (fixed by photosynthesis) from orchid to mycorrhizal symbiont was observed and the results from Chapter 5, one can confidently propose the mutualistic nature of the relationship between *M. media* and its mycorrhizal symbiont, *T. calospora*. In addition,
the accumulation of $^{14}$C at the edges of the mycorrhizal network (Figure 6.6), which corresponds to the sites of active fungal growth, suggests that orchid derived carbon was actively being utilised by the mycorrhizal symbiont to fuel its own growth, as reported in other mycorrhizal systems (Bevege et al., 1975; Morton & Benny, 1990; Cairney & Alexander, 1992). Alternatively, this accumulation of $^{14}$C at the actively growing edges of the mycorrhizal network could be a consequence of nutrient uptake (occurring at those sites), as nutrient assimilation (nitrogen and/or phosphorus) within fungi have been suggested to be energetically expensive (Bidartondo et al., 2001).

Results of this investigation, when combined with the results of Cameron et al. (2006 and 2008), suggest that orchid mycorrhiza do not operate differently to other mutualistic mycorrhizal systems, in which host derived carbon is exchanged for fungal derived nutrients (Smith & Read, 2008). Although the movement of carbon from green *M. media* to its mycorrhizal symbiont has been demonstrated, no quantification of $^{14}$C was carried out as the aim of this study was to establish the occurrence of carbon flow from *M. media* to its mycorrhizal symbiont. It would be essential to conduct further in depth studies, where $^{14}$C values are quantified, in order to determine how much of the orchid photosynthate is transferred into the mycorrhizal symbiont, in order to establish the carbon ‘costs’ associated with forming the symbioses with *T. calospora*.

### 6.4.3 Inter-generational sharing of carbon, from photosynthetic adult into developing non-photosynthetic protocorms of *M. media*, by way of the mycorrhizal network

Results of this investigation have shown carbon, originally supplied to the mycorrhizal symbiont (by photosynthetic *M. media*), to be transferred into developing non-photosynthetic *M. media* protocorms that share the ‘parental’ mycelial network. This suggests that some form of ‘parental nurture’, or inter-generational sharing of carbon, was occurring in *M. media*, where carbon, after fixation by photosynthesis, was being shared with developing protocorms by way of the mycorrhizal symbiont. Although $^{14}$C contents of these developing protocorms were a few orders of magnitude lower than what was present within the adult plant, values presented in Table 6.8 show that $^{14}$C was indeed being accumulated within these protocorms.
The sharing of nutrients, between associated partners in common mycorrhizal networks has been reported previously in arbuscular (Lerat et al., 2002; Giovannetti et al., 2004) and ectomycorrhizal systems (Simard et al., 1997a; Simard et al., 1997b; McKendrick et al., 2000). Specifically, results of this investigation were comparable to what was reported by McKendrick et al (2000), in which the transfer of $^{14}$C from non-orchid plants (Betula pendula and Salix repens) to developing protocorms of the myco-heterotrophic orchid Corallorrhiza trifida was observed to occur by way of a common ectomycorrhizal network. However, given that McKendrick et al (2000) was fundamentally working on an ectomycorrhizal system, this study is the first to show the inter-generational sharing of carbon between green M. media and developing non-photosynthetic M media protocorms, by way of the orchid mycorrhizal symbiont.

This inter-generational sharing of carbon also provides a plausible explanation for the common observation of orchid recruitment occurring in the vicinity of established plants (Dixon, 1991; Rasmussen, 1995). Studies investigating orchid recruitment have suggested that recruitment of orchid seedlings is heavily influenced by the presence of adults, with the probability of successful orchid (terrestrial) seed germination being inversely correlated to increasing distances from established adult plants (Batty et al., 2001; Diez, 2007; Jacquemyn et al., 2007; Jacquemyn et al., 2012; McCormick & Jacquemyn, 2014). In contrast, germination of orchid seeds which have been dispersed beyond this ‘optimal zone for germination’ were more dependent on inherent soil conditions such as nutrient availability and pH (Batty et al., 2001; Diez, 2007; McCormick & Jacquemyn, 2014).

The observations made in these parental nurture experiments are of considerable significance. Clearly, they need to be repeated, with increased levels of replication and using a greater variety of orchid-fungus combinations.
6.5 Conclusions

Results of this chapter have elucidated aspects of the bi-directional flow of carbon between *M. media* and its mycorrhizal symbionts. Important observations include:

- Cellulose functions as a likely natural source of carbon for developing *M. media* protocorms
  - As indicated by the assimilation from $^{14}$C labelled cellulose and subsequent transfer of $^{14}$C labelled product into developing non-photosynthetic *M. media* protocorms.
    - Demonstrates the ‘up-flow’ of carbon from fungus to orchid, thus establishing the importance of the mycorrhizal association for the successful germination of *M. media*.
  - The ability to degrade labelled cellulose indicates the saprophytic ability of *T. calospora*.
- The mutualistic nature of the relationship between *M. media* and *T. calospora* has been established
  - By the movement of carbon from orchid to the mycorrhizal symbiont.
- Potential for the facilitation by the fungal symbiont, of inter-generational sharing of carbon in *M. media*.
  - As demonstrated by the movement of $^{14}$C from orchid, into mycorrhizal network and ultimately into developing protocorms of *M. media*.
  - Potentially explaining why recruitment of *M. media* predominantly occurred in the vicinity of adult plants.

Observations from this chapter provide support for HYPOTHESIS 5: That the mycorrhizal fungi of *M. media* are involved in: a) As donors of carbon in the below ground heterotrophic phases of *M. media* development, and b) As recipients and translocators of C from adults in their autotrophic phases of growth, to the soil, and potentially to developing heterotrophic protocorms.
6.6 References


Chapter 7: General Discussion
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This is the first study of the physiological ecology of the common mignonette orchid, *M. media*, and is amongst the first to consider the mineral nutrition of Australian orchids. It has established the involvement of mycorrhizal fungi, both in the capture and transport of phosphorus (P) to the plant, and in the provision of carbon (C) for the developing protocorm. In addition, the provision of C has also been shown to occur from photosynthetically active plant, to the vegetative mycelium of the mycorrhizal symbiont and even onwards to support germinating seeds in the vicinity of the mycelial network.

The observations concerning the capture and transport of P may be of greatest significance in the context of the ability of this orchid to establish both in its natural habitats, and in disturbed habitats where it colonizes as a ruderal. Over its natural range, the orchid occurs extensively on two soil types, the Bassendean and the Spearwood, both of which are derived from ancient sand dune systems, and from which all of the major mineral nutrients have been leached over the millennia (McArthur, 1991; Brown *et al.*, 2008). Amongst these nutrients, P is widely acknowledged to be the one to critically limit plant growth (Laliberte *et al.*, 2012). Early analyses of the total P contents of these soils (McArthur, 1991) revealed (Table 7.1) that extreme depletion of the element in the mineral horizons. However, quantities of P were found to range from 18 to 22 mg g\(^{-1}\) in the surface layers of the soil profile that were organically enriched (soil organic layer, highlighted in bold in Table 7.1).

In terms of ‘plant available’ P, Laliberte *et al.* (2012), using anion exchange membranes, detected less than 0.5 mg P per kg soil in these ancient soil systems. These values are amongst the lowest recorded worldwide. While studies within the plant families that dominate these ecosystems have identified a number of mechanisms such as selective allocation of P to seed reserves (Kuo *et al.*, 1982; Pate & Dell, 1984; Lamont & Groom, 2002; Denton *et al.*, 2007) and modification of root anatomy (Lamont, 1974; Shane *et al.*, 2004; Shane *et al.*, 2005; Lambers *et al.*, 2006; Playsted *et al.*, 2006) that may have been favourably selected in response to these circumstances (Lambers *et al.*, 2006; Lambers *et al.*, 2008; Lambers *et al.*, 2010; Lambers, 2014), no such analyses have been carried out on the orchids that are also successful colonists of these P-stressed environments. Indeed, seeds of orchids like *M. media*, despite some evidence provided in Chapter 5 that there may be preferential allocation of P to their seeds, are essentially lacking in nutrient ‘reserves’.
Table 7.1. Soil profiles of the two main kinds of dune systems (a: Bassendean dune system and b: Spearwood dune system) found within the natural distribution of *M. media*, with the corresponding total phosphorus (mg g⁻¹) content of the various soil horizons. Adapted from McArthur (1991).

a: Bassendean dune system

<table>
<thead>
<tr>
<th>Soil depth (cm)</th>
<th>Soil colour</th>
<th>Total phosphorus (mg g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 – 7</td>
<td>Black</td>
<td>18</td>
</tr>
<tr>
<td>7 – 25</td>
<td>Dark greyish brown</td>
<td>10</td>
</tr>
<tr>
<td>25 – 50</td>
<td>Very pale brown</td>
<td>6</td>
</tr>
<tr>
<td>50 – 90</td>
<td>Light yellowish brown</td>
<td>7</td>
</tr>
<tr>
<td>90 – 140</td>
<td>Yellowish brown</td>
<td>9</td>
</tr>
<tr>
<td>140 – 180</td>
<td>Yellowish brown</td>
<td>14</td>
</tr>
</tbody>
</table>

b: Spearwood dune system

<table>
<thead>
<tr>
<th>Soil depth (cm)</th>
<th>Soil colour</th>
<th>Total phosphorus (mg g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 – 6</td>
<td>Very dark grey</td>
<td>22</td>
</tr>
<tr>
<td>6 – 15</td>
<td>Pale brown</td>
<td>12</td>
</tr>
<tr>
<td>15 – 35</td>
<td>Very pale brown</td>
<td>12</td>
</tr>
<tr>
<td>35 – 50</td>
<td>Brownish yellow</td>
<td>14</td>
</tr>
<tr>
<td>50 – 80</td>
<td>Brownish yellow</td>
<td>12</td>
</tr>
<tr>
<td>80 – 110</td>
<td>Yellow</td>
<td>9</td>
</tr>
</tbody>
</table>

A number of observations made in the present study are indicative of a likely role of mycorrhizal fungi in enabling *M. media* to establish under these challenging conditions. Probably the most critical challenge for a minute and essentially nutrient reserve-free orchid seed as it arrives at the soil surface will be to establish a relationship with a mycorrhizal symbiont capable of supporting its successful germination. Several features of the biology of this orchid as revealed in the present study may be conducive to successful early establishment under these stressful conditions.

The first is its compatibility with members of a fungal genus, *Tulasnella*, members of which are acknowledged to have a cosmopolitan distribution as soil saprophytes (Roberts, 1999). As saprophytes, fungi of this kind can be expected to preferentially colonize the organically enriched surface layers of soil, onto which the orchid seeds will be deposited (Roberts, 1999). The chances of early physical proximities of the symbionts are thus increased, and in the event of colonization, the association is formed in a position within the soil profile (the surface organic horizon) with the
highest concentrations of the key limiting mineral nutrient P (Table 7.1), as well as the most critical organic resource, carbon (C). The laboratory studies reported in this thesis confirm the ability of *Tulasnella* species to colonize seeds of *M. media*, and to sustain the development of *M. media* protocorms by the provision of both exogenously supplied P and C. These pathways are shown diagrammatically (coloured arrows) in Figure 7.1.

**Figure 7.1.** Overview of the flow of carbon (atmospheric carbon: blue arrows, fungal assimilated carbon: brown arrow) and phosphorus (red arrows) in *M. media*. The movement of photosynthetic fixed atmospheric carbon (CO₂) from *M. media*, into its mycorrhizal network has been established. In addition, the movement of this carbon has been tracked into developing *M. media* protocorms. The assimilation and uptake of both carbon and phosphorus by the mycorrhizal symbiont is indicated by brown and red arrows respectively, and highlights their subsequent transfer into developing *M. media* protocorms, seedlings and adults. Movement of fungal assimilated carbon into green *M. media* plants have yet to be determined, and are indicated with hollow brown arrows.

A further feature of the biology of *M. media* that may contribute to its ability to develop successfully in these regions of the soil profile is its propensity to produce lateral and/or upwardly growing mycorrhizal roots (see photographs of two year old seedlings harvested from the field in Figure 7.2). This negative geotropism will enable
the absorptive system of the plant to retain its position in the relatively P and C enriched surface horizon of the soil. The inherently higher water holding capacity of the organic matter may be an additional benefit, both in terms of tissue hydration in both partners of the symbiosis, and the ability to mobilise nutrients from organic resources. Seen in terms of the chronology of plant development, it can be envisaged that early colonization in the soil surface layers by a widely occurring compatible soil saprophyte enables growth of the essentially heterotrophic protocorm at or close to the soil surface (Figure 7.1). Within the laboratory, this developmental sequence may even proceed to the production of seeds within the first year.

Figure 7.2 Development of second year *M. media* tubers excavated from field conditions, highlighting the development of upward pointing/negative geotropic roots. **a)** Initial resprouting of dormant tuber, two – three weeks after the onset of autumn rains. **b)** Similar aged tuber, four weeks after resprouting. **c)** Similar aged tuber, six weeks after resprouting.

It appears that in the field, the protocorm (at the end of its growing season) produces a descending ‘root’ or ‘dropper’ a few centimetres in length, at the distal end of which the nutrient enriched tuber (supporting the resprouting of the plant in the second year) develops, still within the organic horizon. It is from this tuber that the mycorrhizal roots develop in the second year, with fungi emerging from these roots colonizing the surrounding environment (Figure 7.1). In addition, this distal formation of tubers corroborates the observations of a previous study, which reported the distal formation of daughter tubers in *Microtis* (Dixon, 1991).
Carbon, probably derived from complex organic residues including cellulose, of the uppermost soil horizon, along with P, essentially moves towards the developing orchid protocorm (Figure 7.1, brown arrows), in what has been termed the ‘upward’ direction (Cameron et al., 2008). The present study confirms, however, that once an autotrophic shoot is present, the soil borne network of fungal mycelium can be supplied with C fixed by photosynthesis, and directed ‘downwards’ into the soil (Figure 7.1, blue arrows). The extent to which this downward pathway enables the fungal symbiont to become independent of soil C reserves remains to be determined. To conservatively extrapolate the C results obtained in this present microcosm study, it will not be surprising if future studies (carried out with habitat soil, or under natural habitats) show additional sources of C (eg: photosynthate from other mycorrhizal plants) being ‘tapped’ or utilised by orchid mycorrhiza, and that the observations of this study only represent a small proportion of the overall carbon cycle, given that the carbon cycle in other mycorrhizal systems have been shown to be a highly dynamic process which occurs both at the intra- and inter-species level (Smith & Read, 2008).

The C and P assimilation pathways of *M. media*, as revealed by this study, and summarized in Figure 7.1, demonstrate the critical attributes of the mycorrhizal association of this orchid. The extent to which these mycorrhizal pathways, although described for C (and not the critical nutrient P) in a few Australian orchids (Bougoure et al., 2010; Dearnaley & Bougoure, 2010; Sommer et al., 2012), and for C and P in a small number of European species (Cameron et al., 2007; Cameron et al., 2008; Liebel et al., 2010), contribute directly to the success of *M. media* as an aggressive and invasive species remains to be fully explored. Unfortunately, due to the way the C and P experiments were set up (as two different independent studies), no further extrapolation of these results could be confidently undertaken to discuss the reciprocal exchange of C and P. However, building on these preliminary studies, it should be relatively easy to design and carry out future studies investigating the effects of C on P uptake, and vice versa. As emphasized by Dearnaley et al. (2012), who highlighted the critical need for experimental analysis to determine the ‘upwards’ and ‘downwards’ flow of nutrients within the orchid mycorrhizal system, any future exploration with *M. media* should be based firmly upon experimental determination of the functional relationships of the symbionts.
Interestingly, mycorrhizal specificity of the kind apparently shown by _M. media_, has been suggested as a main driver of both expansion and of rarity in Australian orchids (Hollick _et al._, 2005; Bonnardeaux _et al._, 2007; Bougoure _et al._, 2009). Thus, several other widespread ruderal species, notably _Pterostylis nutans_ R.Br, _P. sanguinea_ D.J. Jones & M. Clements, _P. longiflora_ R.Br, _P. obtusa_ R.Br associate specifically with _Ceratobasidium_ (Bougoure _et al._, 2005; Bonnardeaux _et al._, 2007), while the highly successful alien ruderal from South Africa, _Disa braceata_, is said, like _M. media_, to associate exclusively with _Tulasnella_ spp. (Bonnardeaux _et al._, 2007). Bonnardeaux _et al._ (2007), argue that the benefits of specificity (in orchids), at least to a fungal genus, probably outweighs the disadvantages, provided that the targeted symbiont has, as in the case of _Tulasnella_, a very widespread distribution.

Clearly, the extent of abundance, as of the rarity of any orchid, will be influenced by multiple factors, many of which could not be examined in the course of this present study. However, a major stumbling block in considerations of issues that might determine the ability of any orchid species to successfully colonize an environment has been a lack of knowledge in the fundamentals of their nutrition. The present study provides a step towards the understanding of the role of mycorrhizal associations in facilitating the colonization of an often alien environment by a successful orchid species.
7.4 References


Hollick PS, Taylor RJ, McComb JA, Dixon KW. 2005. If orchid mycorrhizal fungi are so specific, how do natural hybrids cope? Selbyana 26 (1,2): 159-170.


Appendix
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Appendix 1

A.1.1 Materials and methods - sampling and identification of mycorrhizal symbionts from adult *M. media* plants utilised in microcosm experiments.

In order to establish the molecular identities of the mycorrhizal symbionts associating with the adult *M. media* plants utilised in the microcosms, 5mm cubes of agar (3 cubes per microcosm) were removed from around the roots (containing the mycorrhizal symbionts) after the initial two week incubation period, and plated onto fungal isolation media [FIM; 0.3 g⁻¹L sodium nitrate, 0.2 g⁻¹L potassium dihydrogenorthophosphate, 0.1 g⁻¹L magnesium sulphate, 0.1 g⁻¹L potassium chloride, 0.1 g⁻¹L yeast extract, 2.5 g⁻¹L sucrose, 9 g⁻¹L agar, pH 6.8 before autoclaving, 10 mL of streptomycin sulphate stock (0.14 g of streptomycin salt in 70 ml of sterile distilled water) solution added after autoclaving]. Fungal isolates were sub-cultured and screened as per conditions outlined in Chapter 4, and a total of 21 fungal isolates were established. Molecular identities of these isolates were investigated with two primer pairs ITS1-F (5’-CTT GGT CAT TTA GAG GAA GTA A-3’) & ITS4 (5’-TCC TCC GCT TAT TGA TAT GC-3’), and ITS1-F (5’-CTT GGT CAT TTA GAG GAA GTA A-3’) & ITS4-Tul (5’-TCC GTA GGT GAA CCT GCG G -3’) (Taylor & Bruns, 1999; Taylor & McCormick, 2008; Phillips *et al.*, 2011). For PCR and sequencing conditions, please refer to Chapter 4.

A.1.2 Results

The majority of fungal isolates derived from adult *M. media* introduced into microcosms have been identified as *Tulasnella* species (Table 1.1), with the exception of one *Fusarium* species.
Table A1.1 Putative identities of 21 fungal isolates established from adult *M. media* plants introduced into microcosms, with the corresponding primers utilised (+ : PCR amplification of genomic extract, - : no PCR amplification of genomic extract). Percentage homology, accession number (on Genebank) and their respective publications are presented. Fungal isolates (from this present study) which corresponded to sequences submitted directly to Genebank (unpublished) are highlighted as ‘direct input’.

<table>
<thead>
<tr>
<th>Fungal isolate</th>
<th>Primer combination: ITS1-F and ITS4</th>
<th>Primer combination: ITS1-F and ITS4-Tul</th>
<th>BLAST homology (%)</th>
<th>Closest relative</th>
<th>Accession number</th>
<th>Publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR 124</td>
<td>-</td>
<td>+</td>
<td>98</td>
<td><em>Tulasnella</em> sp.</td>
<td>AY 373266.1</td>
<td>(McCormick <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td>DR 125</td>
<td>-</td>
<td>+</td>
<td>98</td>
<td><em>Tulasnella</em> sp.</td>
<td>AY 373266.1</td>
<td>(McCormick <em>et al.</em>, 2004)</td>
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<td>(Suárez <em>et al.</em>, 2006)</td>
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“Fungi are not enemies of plants, but are their essential partners”

- Sir David Attenborough (Kingdom of Plants, 2012)